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The aim of this study was to test vaccines expressing mouse mutant or wild-type p53 for induction of protective immunity against challenge with tumor cell lines expressing either mutant or high levels of wild-type p53. Our goal is to develop an efficacious vaccine with broad applicability for the treatment of human breast cancer patients. The completed studies show that vaccinia virus recombinants expressing full-length wild-type p53 provide up to 70% protection to one of the model tumor cell lines investigated. Vaccine efficacy can be improved by using mouse IL-12 as an adjuvant. This combination treatment also results in complete tumor regression in approximately 40% of mice carrying already established tumors. Tumor rejection (with or without IL-12) is mediated by CD4+ and CB8+ T cells as well as by NK cells. The vaccine was less efficacious against some other tumor cell lines that expressed mutant p53 or high levels of mutant wild-type p53. DNA vaccines expressing varied forms of wild-type or mutant p53 were also shown to inhibit the growth of some but not all tumors overexpressing p53. The efficacy of the DNA vaccines was improved by expressing the protein as a fusion construct with an adenoviral leader sequence.

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TABLE OF CONTENTS	PAGE NUMBER

(5) INTRODUCTION

Vaccination is the most effective medical intervention to reduce human morbidity and mortality. While vaccines to microbial agents have been used successfully for decades to reduce the incidence of infectious diseases, efficacious vaccines to cancer have been scarce, partially due to a lack of suitable 'tumor specific' antigens. With advances in molecular oncology, protein alterations are being identified at a rapid rate in transformed cells. These modifications can be caused by point mutations, alternate splicing or overexpression of normal gene products.

One of the most frequent alterations of human tumors are mutations or marked overexpression of the p53 protein, either of which can be found in more than 50% of the most common types of human cancers. Wild-type p53 serves as a tumor suppressor protein which regulates cell growth and induces apoptotic cell death upon severe cellular DNA damage. Mutations of p53, which cluster in well-defined hot spots of the gene, lead to structural changes and functional inactivation of the p53 protein by altering its DNA binding domain. The p53 protein is only functionally active upon formation of tetramers, mutations of a single p53 allele can therefore have a dominant negative effect on the correct protein synthesized by the wild-type allele. Most mutations also prolong the half-live of the protein resulting in overexpression.

Point mutations of self proteins can induce an immune response provided the amino acid exchanges are flanked by anchoring residues that are able to bind to MHC determinants. Peptides delineated from a mutated region of p53 were shown to induce in experimental animals a T cell mediated immune response to tumors expressing the homologous mutation (1,2). The immune response to individual epitopes is genetically restricted (3), thus limiting the usefulness of single epitope vaccines for outbred populations.

Overexpression of a self protein can cause exposure of so-called cryptic epitopes (4,5). T cells are negatively selected in the thymus during development, causing apoptotic cell death of T cells which carry receptors with high affinity to self epitopes. Additional extrathymic pathways ensure tolerance to immunodominant epitopes of self proteins. Cryptic epitopes are epitopes that at physiological expression levels have too low an affinity for major histocompatibility complex (MHC) determinants to reach the threshold needed for induction of T cell tolerance. Upon over-expression of the protein or upon alteration of its processing, a sufficient amount of these low affine epitopes can associate with MHC determinants and, provided that presentation is mediated by professional antigen presenting cells within the context of lymphatic tissue, result in the induction of a T cell-mediated immune response.

Wild-type p53, a protein that is present at low levels in all nucleated cells throughout the body, was shown by several groups including ours within the realm of this program to induce a T cell-mediated immune response (6-8) in experimental animals upon expression by a vaccine. These findings suggest that wild-type p53 might serve as a highly suitable target antigen for active immunotherapy against a wide variety of tumors.

Different types of subunit vaccines, i.e., vaccines expressing a single antigen or a fragment thereof, can be developed. Recombinant viral vaccines, such as those based on vaccinia viruses (9), generally induce potent T and B cell-mediated immune responses against the inserted antigen. DNA vaccines, one of the latest additions to the field of vaccinology, also result in stimulation of antibodies, T helper cells, and cytolytic T cells (10-13). DNA vaccines were shown to induce T cell responses in non-responder haplotypes (14), indicating that antigen as expressed by a DNA vaccine might cause exposure of hidden (cryptic) epitopes which might be an advantage for vaccines to tumor-associated antigens such as p53.

Within the realm of this program we have tested DNA vaccines and vaccinia virus recombinants expressing wild-type or mutant p53 for induction of protective immunity to challenge

with tumor cells expressing either mutant p53 or high levels of wild-type p53. To further enhance the immunogenicity of the vaccines, we explored the use of p53 that had been modified to allow for more ready association of epitopic peptides with determinants of the MHC. The immune effector mechanisms that are involved in providing tumor rejection in vaccinated animals were analysed by immunohistochemistry, by using genetically engineered mice or by in vivo antibody-mediated depletion of effector cell populations.

(6) BODY

METHODOLOGY

Mice: Female C57Bl/6 and Balb/c mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained at the Animal Facility of The Wistar Institute. Knock-out mice were bred at The Wistar Institute's Animal Facility. A number of these mice were checked annually by PCR conducted with blood lymphocytes for presence of the defect and for homozygocity.

Cells: GL261, a murine glioma cell line of C57Bl/6 origin, and CT-26, a murine colon adenocarcinoma cell line of Balb/c background, were provided by The National Cancer Institute (Frederick, MD). The B16.F10 melanoma cell line was obtained from the American Type Culture Collection (ATCC). The 410.4 and 66.1 mammary adenocarcinoma cell lines were kindly provided by Dr. Amy Fulton, University of Maryland (Baltimore, MD). C57Bl/6 fibroblasts were established from C57Bl/6 embryos at 16-18 days of gestation. (10)1 is a Balb/c fibroblast line with a spontaneous loss mutation of both p53 alleles. The t(10)1ras.E7E6 tumor cell line was generated by stable transfection of (10)1 cells using a triple promoter vector expressing v-Harvey-ras under the control of the LTR, the E7 protein of human papilloma virus (HPV)-16 under the herpes simplex virus thymidine kinase promoter and the E6 protein of HPV-16 under the SV40 promoter. Cells were grown *in vitro* in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics.

A B cell hybridoma secreting IgM to phosphorylated p53 was generated by immunizing C3H/He mice 3 times in 14 day intervals with a peptide composed of a phosphorylated p53 sequence linked to 31D, a T helper cell epitope of the rabies virus nucleoprotein. Splenocytes were fused 5 days after the last immunization to SP2O cells. Hybridomas were selected in HAT medium and tested for presence of antibodies to p53 by an ELISA on plates coated with phosphorylated or unphosphorylated p53 peptide. A positive colony was identfied, subcloned, and isotyped. Ascitic fluid was generated in pristane primed SCID mice. The specificity of the antibody was confirmed by Western Blot analysis and mass spectrophotometry.

Expression of p53: The expression of p53 mRNA by tumor cells was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). PCR products were sequenced at the Nucleic Acid Facility at The Wistar Institute using a dideoxynucleotide termination reaction. Expression of the p53 protein was determined by immunostaining of cell monolayers with the p53-specific monoclonal antibody (mAb) Ab-1 (Oncogene Inc. Cambridge, MA) followed with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin (Ig). Counterstaining of nuclear DNA was performed with propidium iodide. Cells were analyzed by confocal microscopy at the Morphology Core Facility of The Wistar Institute.

Expression of MHC class I determinants: Cells were tested for MHC class I expression by incubating 2 x 10^5 cells with 20 µl of hybridoma culture supernatant containing the mAb SF1-1.1.1 (ATCC) with specificity for K^d or the mAb 28-14-8S (most kindly provided by Dr. E. Heber-Katz, The Wistar Institute) which react with both D^b and L^d for 45 minutes on ice followed by a 30 minute incubation with an FITC-labeled goat anti-mouse Ig antibody. Cells were subsequently analyzed in a fluorescent activated cell sorter (FACS).

Secretion of cytokines: The 24 hr supernatants of tumor cells ($2x10^5$/ml) were assayed for active and latent forms of TGF-ß and for IL-3/GM-CSF using biological assay systems.

Vaccinia virus recombinants: The Vp53-wt recombinant was constructed as follows. The DNA fragment encoding mouse full-length wild-type *p53* (p53-wt) was excised from the pGEM-*p53* vector (kindly provided by Dr. Thanos D. Halazonetis, The Wistar Institute) and cloned into the pSC11 transfer vector for vaccinia virus. A recombinant vaccinia virus containing the *p53*-wt sequence was generated by homologous recombination with vaccinia virus strain Copenhagen using thymidine kinase-negative cells (TK^-). The recombinant viruses were characterized for the presence of *p53* sequence using PCR. Expression of the p53 protein was determined by Western blot analysis of p53 negative (10)1 fibroblasts infected with the vaccinia virus recombinant using commercially available mAbs to p53 (Ab-1 and Ab-3). The vaccinia rabies virus glycoprotein (VRG) recombinant, which is also based on the Copenhagen strain of vaccinia virus, has been described in detail earlier. The vaccinia virus recombinants were purified by high speed centrifugation onto a 36% sucrose cushion and titrated on HeLa or thymidine kinase deficient cells.

DNA vaccines: Expression vectors were generated by cloning different forms of mouse p53 cDNA in various expression vectors. Appropriate expression was tested by immunofluorescence of Western blot analysis. Vectors expressing fusion proteins of p53 were generated by sequential cloning of PCR products of the p53 cDNA and the fusion partner's cDNA into an expression vector. The inserts were sequenced and then tested by staining for p53. In more detail, a pGEM type plasmid (T. Halazonetis, The Wistar Institute) which carries *p53* with a single mutation in position 338 (with reference to the encoded amino acid) and a pSC11 vector (L. Eisenlohr, Jefferson University) which carries the signal sequence of adenovirus human strain 2 were obtained. We designed oligonucleotide primers containing 5' and 3' terminal restriction enzyme sites to amplify the p53 sequence. The PCR product upon restriction enzyme digest was to be cloned into the pSC11 vector just 3' of the signal sequence. The fusion gene was excised from the pSC11 vector and inserted into the multicloning site of the pVR1012 vector. The original pVR1012 vector constructed for optimized expression of proteins under the control of the CMV promoter contains the kanamycin resistance gene. We replaced the kanamycin resistance gene with the ampicillin resistance gene, which contains 2 palindromic AACGTT sequences which have been described to provide an adjuvant effect to DNA vaccines. The resulting construct, upon initial testing by restriction enzyme digest, was sequenced to ensure in frame insertion and faithful PCR amplification of the p53 gene. Transfectants were analyzed by indirect immunofluorescence and confocal microscopy to determine localization of the protein mAb-1 and by Western Blot using mAb-18. A vector expressing the p53 as a fusion protein with ubiquitin was constructed using similar methodologies.

E1-deleted adenoviral recombinant: We generated this recombinant which will be used for in vitro restimulation of cytolytic T cells to p53 and for prime-boost vaccine experiments. A transfer vector carrying mouse p53 fused in frame to the leader sequence of mouse granulozyte macrophage colony stimulating factor was generated. Adenoviral DNA was purified and cut with Cla I to remove the E1 domain containg the cDNA encoding green fluorescent protein. 293 cells were co-transfected with the cut adenoviral DNA and linerarized transfer vector. Plates were overlaid 24 hrs later and then again 5 days later with agarose. Plaques once visible (8-10 days after transfection) were screened under a fluorescent microscope. Several non-fluorescent plaques were harvested, subcloned 3 times and then tested by PCR for p53. A PCR positive plaque was expanded upon infection of cells tested by immunohistochemistry for expression of p53. The protein could be readily identified within the nucleus as well as within the cytoplasma.

Cytokines: Mouse rIL-12 was kindly provided by Dr. Giorgio Trinchieri at The Wistar Institute. For *in vivo* administration, rIL-12 was diluted in phosphate buffered saline (PBS).

Immunization and challenge of mice: Tumor cell lines were titrated in syngeneic mice by subcutaneous (s.c.) inoculation to establish the minimal tumorigenic dose (TD_{100}) for each cell line, defined as the number of cells required for tumor formation in 100% of animals within ~15 to 30 days after inoculation. Protection studies were carried out by injecting 6 to 12 week-old mice s.c. with a single dose of 2×10^7 plaque forming units (pfu) of Vp53-wt virus in 200 µl of saline. Control mice were immunized with the same dose of VRG virus. Mice were challenged 2 or 3 weeks later s.c. with tumor cells. Treatment with rIL-12 (0.25 µg/daily) given intraperitoneally (i.p.) for 4-5 days was initiated at various days post-challenge as indicated. Animals were monitored three times per week for development of tumors, and tumor size was measured. Mice which developed tumors >1.5 cm in diameter or showed signs of functional impairment due to the tumor mass were euthanized according to Institutional Animal Care and Use Committee (IACUC) guidelines.

Treatment of established tumors was performed in animals 14-20 days after s.c. challenge with 1.5×10^5 GL261 cells. Animals were randomized with respect to tumor size (0.2-0.5 mm diameter) before receiving virus and rIL-12 treatment. DNA vaccines were given 2-3 times at 50-100 µg in saline i.m. Mice were challenged 2 -10 weeks after the last injection.

Antibody depletion studies: *In vivo* depletion of specific T cell subsets was accomplished by injecting mice i.p. with rat mAbs specific for mouse CD4 (i.e., GK1.5, ATCC) or CD8 (i.e., 53.6.7, ATCC) using 0.2 ml aliquots of a 1:10 dilution of antibody derived from the ascitic fluid of mice injected with hybridoma cells. The antibodies were injected on days -4, -1, +1, +4 and +8 relative to tumor cell injection (day 0). Preliminary experiments had shown that this treatment resulted in >95% reduction of the appropriate T cell subset. Deletion of natural killer (NK) cells was achieved using rabbit serum against asialo-GM1 ganglioside (Wako Inc., TX) at the dose recommended by the manufacturer. Control mice were inoculated with the same dose of a rat anti-rabbit serum.

Immunohistochemistry: Frozen sections were stained using the avidin-biotin-peroxidase Vectastain Elite ABC kit (Vector Laboratories Burlingame, CA). Sections were developed with 3',3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO.) and counterstained with 1% hematoxilin (HE). Sections were analyzed in a SONY up-5500/5600 microscope with the computational ability to count the number of stained cells per section and to determine the percentage of peroxidase stained area/total tissue area.

RESULTS:

Tumor cell systems: A number of tumor cell lines were obtained. Transcripts of *p53* were amplified by reverse transcription polymerase chain reaction (RT-PCR) and sequenced to characterize potential mutations (Table 1). In addition, for some of the cell lines the levels of p53 expression were established by indirect immunofluorescence or by Western Blot analysis using a p53 specific monoclonal antibody (Ab-1, Oncogene, Cambridge, MA or mAb-18). All of the tumor cell lines that were positive for p53 expressed substantially higher amounts compared to primary fibroblasts. Cell lines were titrated in syngeneic mice upon s.c. inoculation of graded numbers of cells to establish the minimal tumorigenic dose (TD_{100}), defined as the number of cells that causes visible tumors in 100 % of control mice within 2-4 weeks. Some of the cell lines were characterized for secretion of TGF-ß (experiment conducted by Dr. U. Rodeck, The Wistar Institute), IL-3/GM-CSF using an indicator cell system or IL-10 by an ELISA. Cell lines were also tested for expression of MHC class I determinants using a monoclonal antibody reactive to D^b and L^d. Experiments to determine expression of MHC class II determinants are ongoing. The results are summarized in Table 1. We obtained a fibroblast line, termed (10)1, immortalized due to the spontaneous loss of both p53 alleles. This fibroblast line was transformed by a triple promoter

retroviral vector expressing v-Ha-ras under the control of the long terminal repeat, E7 of HPV-16 under the control of the HSV promoter and E6 of HPV-16 under the control of the SV40 promoter. Transformed cells, termed t(10)1rasE7E6, were selected in vivo by passage in mice. This tumor cell line which lacks p53 expression is being used as a control.

Table 1

Tumor line	Tumor type/Origin	p53 Type/Level	1 TD_{100}	TGF-ß secret. act./total (pg/ml)	GM-CSF secretion	MHC expr.
Gl261	Glioma/C57Bl/6	wt/++	1×10^5	70/936	+/-	+
B16.F10	Melanoma/C57Bl/6	wt/n.t.	5×10^4	14/176	n.t.	+
CT-26	Colo-Rectal CA/Balb/c	wt/++	5×10^4	1/2075	+	+
MethA-34	Fibrosarcoma/Balb/c	mu/++++	1×10^6	n.t.	-	++
66.1	Mammary CA/Balb/c	wt/+++	4×10^4	27/759	+++	++
410.4	Mammary CA/Balb/c	wt/++	3×10^4	51/3352	+++	+
t(10)1ras E7E6	Transformed/Balb/c fibroblasts	none	2×10^4	n.t.	+++	+/-

wt* - wild-type *p53*, mu* - mutant *p53*, double mutations causing amino acid exchanges in position 168 (Glu to Gly) and 234 (Met to Isoleucin), 1 TD_{100} - minimal dose of tumor cells that causes visible tumors within 2-4 weeks after subcutaneous inoculation of cells. Cells were tested for secretion of TGF-ß - active and latent form (40) and for secretion of IL-3/GM-CSF. MHC class I expression was determined by indirect immunofluorescence with an antibody to L^d and D^b followed by FACS analysis.

Vaccines and reagents to p53: Vaccinia virus recombinants expressing the full-length wild-type p53 (termed Vp53-wt) or different mutations (Vp53-mu135 -mutation at position 135 from phenylalanine to alanine, Vp53mu168.234 - double mutation at position 168 glutamic acid to glycine, and at position 234 methionine to isoleucin) of p53 were constructed and tested upon infection of p53 negative cells for expression of p53 transcripts by RT-PCR using the appropriate p53 primers and for protein expression by Western Blot analysis. DNA vaccines based on different plasmid vectors using the SV40 or the cytomegalovirus (CMV) promoter, such as the high expressing vector pVR1012.2 (kindly provided by Vical, Inc.) in which we replaced the kanamycin resistance gene with the ampicillin resistance gene (which contains additional immunostimulatory CpG sequences, 17) were generated expressing full-length wild-type, mutant (135, or 168.234) or truncated (deletion of the 1-70 transactivation domain) p53. Additional DNA vaccines expressing a biologically inactive form of p53 as a fusion protein with a leader sequence (to prevent nuclear transport of p53 and to facilitate transport into the endoplasmic reticulum {ER} and potentially into the extracellular domain) were generated, analyzed in vitro and testing in vivo. Some of the in vivo experiments are still ongoing. An E1-deleted adenoviral recombinant expressing a biologically inactive form of mouse p53 was generated and tested in vitro. In vivo protection experiments with this recombinant used either alone or in a prime-boost regimen with the DNA vaccines are ongoing. A recombinant baculovirus expressing mouse p53 upon infection of insect cells, as determined by Western Blot analysis, was produced. A monoclonal immunoglobulin (Ig)M antibody which recognizes a linear epitope of the C terminus of p53 as determined by Western Blot analysis was also generated by using synthetic phosphorylated peptides coupled to a T helper cell epitope. This antibody, generated in collaboration with Dr. L Otvos, termed mAb-18, recognizes a phosphorylated epitope of p53 (18).

Immune response to vaccinia virus recombinants expressing p53: Most of our studies have been based on the Vp53-wt vaccine expressing full-length wild-type mouse p53.

Induction of protective immunity to challenge: Mice immunized with a single dose of 2×10^7 plaque forming units (pfu) of Vp53-wt were partially protected (i.e., 30-80 % complete protection, and delayed onset of tumors in the rest of the mice) against challenge with a minimal tumorigenic dose of the glioma line (GL)261 glioma cell line which caused tumors in 90-100 % of the control mice immunized with 2×10^7 pfu of a vaccinia rabies virus glycoprotein (VRG) recombinant based

on the same strain of vaccinia virus (i.e., Vaccinia virus strain Copenhagen, ref. 9). Vaccination with the Vp53-wt vaccine did not protect against challenge with a 10 times higher dose of GL261 tumor cells. Nevertheless, Vp53-wt vaccinated mice that remained tumor-free after the initial inoculation with 1 TD_{100} were then completely resistant to a subsequent challenge with 10-50 times more tumor cells that rapidly formed tumors in all of the VRG-immune control mice (see appendix and Table 2) but not against challenge with an unrelated p53-expressing tumor cell line. These data show that the tumor challenge had a booster effect in vaccinated mice which had then augmented resistance to further encounter with the same tumor cells. Resistance was even achieved against tumor cell introduced directly into the central nervous system. The efficacy of the Vp53-wt vaccine against growth of the GL261 cell line was not augmented by vaccinating mice with 2 doses of 2 x 10^7 pfu each of the Vp53-wt vaccine (data not shown), which presumably reflects neutralization of the second vaccine dose by antibodies to surface proteins of vaccinia virus. A similar level of protection to GL261 challenge was achieved with a vaccinia virus recombinant expressing a mouse mutant p53 protein (Vp53-mu135, data not shown).

We next tested, in a series of experiments, if vaccination with the Vp53-wt vaccine induced protection against other tumors. No protection could be achieved against the p53-negative tumor cell line, i.e., t(10)1rasE7E6, tested in BALB/c mice. Some protection could be achieved against wild-type p53 positive adenocarcinomas such as CT-26 (colon cancer), 66.1 and 410-4 (mammary tumors also tested in BALB/c mice). Protection was not as impressive as that seen in C57Bl/6 mice challenged with GL261 cells; the vaccine did not induce complete resistance in more than 10-30 % of mice, but resulted in a statistically significant delay of onset of tumor lesions. Lack of protection was not strictly correlated with levels of p53 expression, for example the 66.1 tumor cell line expresses higher levels of p53 than the GL261 cell line. Characteristics of the individual tumor cell lines such as density of MHC class I expression, down-regulation of the transporter associated protein (TAP)-1/-2 or LMP2/7, expression of fas-ligand, and secretion of immunomodulatory molecules such as IL-10 or prostaglandin E (reviewed in 19) might affect the efficacy of the Vp53-wt vaccine.

Table 2

Vaccine	Cell Line	% Resistance	Onset of Tumors (days ± SD)	Significance
VRG	GL261	9.7	28.5±15.1	
Vp53-wt	GL261	76.4	51.3±24.6	0.000001
VRG	B16.F10	0	23.5±4.1	
Vp53-wt	B16.F10	0	30.4±7.8	0.035
VRG	66.1	0	28.0±5.7	
Vp53-wt	66.1	30	45.3±9.1	0.017
VRG	410.4	0	18.9±3.2	
Vp53-wt	410.4	12.5	24.6±1.5	0.008
VRG	CT-26	0	39.8±1.7	
Vp53-wt	CT-26	10	49.6±5.9	0.003
VRG	t(10)1rasE7E6	0	19.2±2.9	0.038
Vp53-wt	t(10)1rasE7E6	0	16.4±1.7	

The table shows the summary of several experiments. Protection to GL261 cells was tested in 5 separate experiments in a total of 31 (VRG) or 34 (Vp53-wt) mice. Groups of mice (minimal number : 8) were immunized with 2 x 10^7 pfu of VRG or Vp53-wt virus. Two weeks later mice were challenged with 1-2 TD_{100} of the different tumor cells. Mice that failed to develop tumors within the observation periods (50-100 days) were scored as resistant. For mice that developed tumors, the mean day from challenge until visible onset of tumor growth (>2 mm in diameter) ± standard deviation was recorded. Significance of difference between onset in VRG and Vp53-wt vaccinated mice was calculated by student τ test.

No correlation was seen between vaccine failures and levels of TGF-ß secretion (Table 1). In addition, genetic difference between the two mouse strains, i.e., C57Bl/6 and BALB/c, are likely to influence vaccine induced resistance. For example, BALB/c mice, due to a postulated defect in the IL-12 pathway, are more prone to develop T helper (Th)2 responses, which might be less effective in eliminating tumor cells. Furthermore, both mouse strains have a different MHC haplotype, which determines availability of suitable T cell epitopes (i.e., Ir-gene control).

Immune effector mechanisms involved in providing protection to tumor challenge:
The immune effector mechanisms that provide protection upon Vp53-wt vaccination were determined in knock-out mice. The following four knock-out mouse strains were used: cluster determinant (CD)4 knock-out (KO) mice (20) to test for a requirement for T helper cells, ß2

microglobin-KO mice (21) that lack MHC class I expression and hence $CD8^+$ T cells to test for a putative role of cytolytic T cells, perforin knock-out mice (22) to determine a role for cytolysis via the perforin pathway, and μMT mice (23), that due to a immunoglobulin heavy chain transmembrane defect lack functional B cells to establish a potential contribution of antibodies in mediating tumor rejection. In addition, we tested Interleukin (IL)-4KO and GKO mice to test for a role of IL-4 indicative for a Th2 response or interferon-gamma suggestive for a Th1 type immune response respectively. Neither CD4KO mice nor ß2KO mice could be protected by the Vp53-wt vaccine against challenge with the GL261 cell line, indicating that both T cell subsets were needed to limit the growth of the GL251 cell line. In contrast, μMt mice showed only a slight reduction in resistance suggesting that antibody-mediated immune effector mechanisms do not play a major role in providing protection. Perforin-KO mice were not protected upon vaccination indicating that perforin-mediated cytolysis is needed for the elimination of GL261 tumor cells (table 3). GKO mice could readily be protected against growth of GL261 cells by vaccination to p53 while IL-4KO mice remained susceptible after vaccination indicating that Th2 type immune mechanisms played a predominant role in providing rejection of tumor cells.

To further ascertain that protection required both $CD8^+$ and $CD4^+$ T cells and to furthermore establish if $CD4^+$ T cells had to be present during the induction phase (i.e., shortly after vaccination) to provide help to $CD8^+$ T cells or if $CD4^+$ T cells were also needed during the effector phase (i.e., at the time of tumor challenge), antibody depletion studies were conducted using the monoclonal antibodies GK1.5 (rat anti CD4) and 53-6-72 (rat anti-CD8). In the initial experiment, mice were treated with the antibodies prior to vaccination. In a subsequent experiment, mice were first vaccinated and then 2 weeks later treated with the antibodies at the time of tumor challenge. Regardless of the timing, depletion of either $CD4^+$ or $CD8^+$ T cells resulted in loss of protection. In addition, mice that were depleted of either subset showed accelerated growth of tumors which was particularly pronounced after depletion of $CD4^+$ T cells. Natural killer cells were also required for limiting tumor growth in Vp53-wt vaccinated GL261-challenged mice as depletion of this subset by treatment with a rabbit serum to asialo-GM-1 completely abrogated protection.

We conducted similar experiments to confirm the importance of Th2 type immune responses by depleting mice of IL-4 or interferon (IFN)-gamma just prior to challenge with tumor cells. Again depletion of IFN had no effect on vaccine induce protection while depletion of IL-4 was shown to be detrimental (data not shown).

Table 3: Protection in mice with genetic defects in immune effector functions.

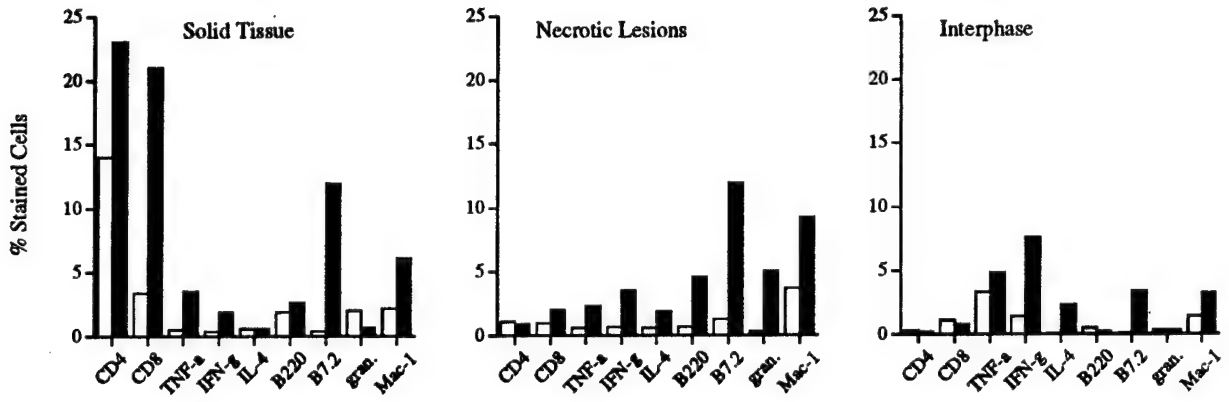
Vaccine	Recipient	Tumor-Free Interval (days ± SD)	% Complete Protection
Experiment I			
VRG	CD4KO	26.0± 6.5	20
Vp53-wt	CD4KO	18.6± 3.1	20
VRG	ß2m	25.5 ± 4.5	0
Vp53-wt	ß2m	26.8 ± 3.4	0
VRG	C57Bl/6	33.3±12.6	30
Vp53-wt	C57Bl/6	38.0± 9.6	60
Experiment II			
VRG	µMt	34.7±11.0	16
Vp53-wt	µMt	43.0±17.0	43
VRG	PKO	27.9± 6.2	0
Vp53-wt	PKO	25.4± 8.1	0
VRG	C57Bl/6	35.0±11.0	12
Vp53-wt	C57Bl/6	41.0± 4.0	44

The table shows the results representative of 2 to 3 experiments. Mice were vaccinated with VRG or Vp53-wt. They were challenged 2 weeks later with GL261 tumor cells and tumor development was recorded. In each experiment control wild-type C57Bl/6 mice were tested in parallel.

Histochemical analysis of tumors: GL261 tumors from VRG or Vp53-wt vaccinated mice were analyzed for an inflammatory infiltrate by staining for cell surface markers on T helper cells (CD4), cytolytic T cells (CD8), B cells (B220), granulocytes, macrophages (mac-1), and activated antigen presenting cells (B7.2) using commercially available antisera or monoclonal antibodies. Frozen sections were stained using the avidin-peroxidase Vectastain Elite BC kit (Vector Laboratories). They were developed with 3',3'-diaminobenzidine tetrahydrochloride (Sigma) and counterstained with 1% hematoxilin eosin (HE). In addition, formalin fixed sections were stained for cytoplasmatic expression of IFN-γ, IL-4 and tumor necrosis factor (TNF)-α. Sections were analyzed in a SONY ultraviolet (uv)-5500/5600 microscope with the computational ability to count the number of stained cells per section or to determine the % area of stained cells/total area of cells. The tumor sections derived from tumors of 1-2 cm in diameter showed three distinct areas: solid tissue which was fairly homogeneously infiltrated with mononuclear cells, necrotic areas with a

more discrete infiltrate, and the interphase between solid and necrotic parts of the tissue which showed the most pronounced aggregation of mononuclear cells. Two to three representative sections were analyzed and the means of the obtained values are shown in Figure 1. Comparing tumors from VRG immune control mice and Vp53-wt vaccinated mice, the most striking observation was the increased influx of $CD8^+$ T cells into the solid tissue. $CD4^+$ T cells, already present at a high level in tumors of control mice, only increased about 2 fold in solid tissue. Only a few T cells were scattered throughout the necrotic area or at the interphase, where granulocytes, mac-1^+ cells, and $B220^+$ cells were more frequent. B7.2, a co-stimulatory molecule expressed on antigen-presenting cells such as dendritic cells, was mainly found in the solid tissue of Vp53-wt vaccinated mice concommittant with $CD4^+$ and $CD8^+$ T cells. Intracytoplasmatic staining for the cytokines, i.e., IFN-γ, IL-4, and TNF-α, showed an increase for all of them, less in solid tissue but mainly at the interphase and in areas of necrotic lesions. The most pronounced increase was seen for IFN-γ. Taken together these data indicate that $CD4^+$ T cells play a major role in controlling tumor growth even without specific vaccination, massive excavation of $CD8^+$ T cells is a consequence of Vp53-wt vaccination which also enhances (presumably as a bystander effect of antigen specific effector mechanism) recruitment of inflammatory cells such as granulocytes and macrophages. Specific vaccination also enhances influx of cytokine secreting cells, especially those secreting IFN-γ, a cytokine indicative for a Th1 type immune response (Figure 1).

Figure 1



Legend: Thin section of 1-2 cm in diameter GL261 tumors from Vp53-wt (■) or VRG (□) immunized mice were stained with antibodies to cell surface markers or cytokines in the cytoplasma. Several 3 sections of each tumor containing solid tissue, necrotic tissue, or the interphase between the two were analysed.

IL-12 has a synergistic effect to vaccination with Vp53-wt: In an effort to enhance vaccine efficacy, IL-12, a cytokine that promotes Th1 type responses and has known therapeutic effects against tumors which might in part be caused by its anti-angiogenic activity, was used as an adjuvant. Vp53-wt or VRG vaccinated mice challenged 2 weeks after immunization were treated at varied times after tumor inoculation with 0.25 μg per mouse per day of mouse recombinant IL-12 protein given i.p. for 4 consecutive days. IL-12 itself (i.e., in combination with the VRG control

vaccine) resulted in partial protection, nevertheless, a combination of Vp53-wt and IL-12 had a synergistic effect causing complete protection in up to 90-100% of mice. The degree of protection depended on the time lapse between tumor challenge and IL-12 therapy. The best results were obtained when IL-12 was given shortly (within 1-6 days) after challenge. Depletion of $CD4^+$, $CD8^+$ or natural killer (NK) cells at the time of tumor challenge resulted in a loss of protection (Table 4).

Mice that were vaccinated with a combination of Vp53-wt and IL-12 or IL-12 only that survived the initial challenge with tumor cells were rechallenged with an increased dose of GL261 cells or with unrelated p53 expressing tumor cells, i.e., B16.F10 cells. Again, mice showed complete protection to challenge with GL261 cells but only partial protection (i.e., delay of onset of lesions) to B16.F10 cells. Mice vaccinated initially with the Vp53-wt vaccine and IL-12 showed the most pronounced protection to B16.F10 cell challenge with regard to mean delay of tumor onset (Table 5).

Table 4

Vaccine	Depletion of lymphocytes	IL-12, days of treatment	experiments 1+3		experiments 2 + 4	
			Tumor free interval (days)	% Protection	Tumor free interval (days)	% Protection
Vp53-wt	-	-	45 ± 16	63	42 ± 15	42
VRG	-	-	45 ± 24	14	23 ± 4	0
Vp53-wt	-	2-5	68 ± 17	75	34 ± 0	**90**
VRG	-	2-5	85 ± 7	75	41 ± 13	20
Vp53-wt	-	5-9	>100	**100**	44 ± 11	30
VRG	-	5-9	42 ±4	50	41 ± 13	40
Vp53-wt	-	15-19	40 ± 0	63	64 ± 17	10
VRG	-	15-19	50 ± 14	42	35 ± 14	20
Vp53-wt	-	-	40 ± 8	30	54 ± 19	30
VRG	-	-	26 ± 8	0	29± 4	0
Vp53-wt	-	2-5	46 ± 0	**90**	>100	**100**
VRG	-	2-5	38 ± 16	70	48 ± 0	77
Vp53-wt	CD4	2-5	23 ± 10	10	21 ± 2	0
VRG	CD4	2-5	25 ± 9	0	17 ± 2	0
Vp53-wt	CD8	2-5	27 ± 10	10	43 ± 11	20
VRG	CD8	2-5	22 ± 8	20	26 ± 7	13
Vp53-wt	CD4/8	2-5	n.t.	-	18 ± 8	0
VRG	CD4/8	2-5	n.t.	-	16 ± 2	0
Vp53-wt	NK	2-5	n.t.	-	26 ± 12	0
VRG	NK	2-5	n.t.	-	38 ± 2	20

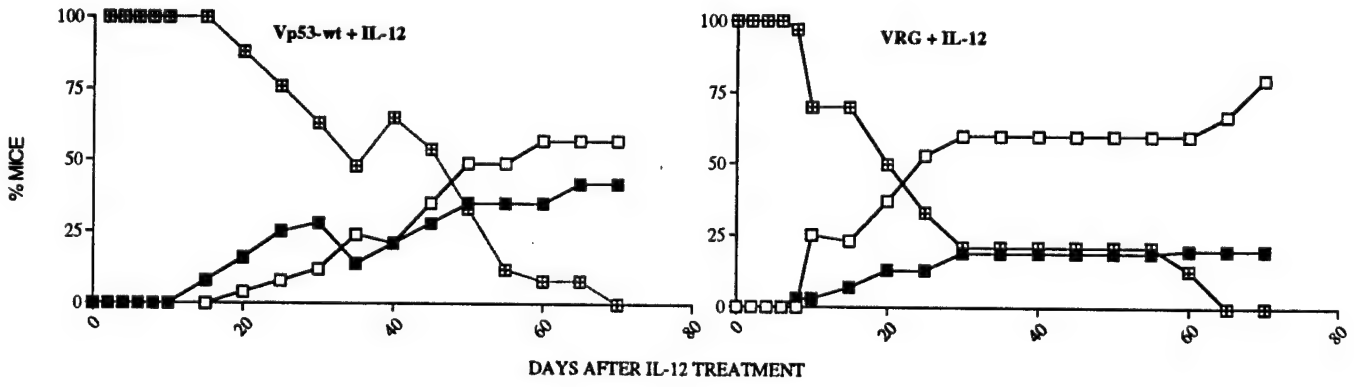
Groups of 8-10 mice were vaccinated with 2 x 10^7 pfu of Vp53-wt or VRG virus. They were challenged 14 days later with 1 TD_{100} of GL261 cells. Onset of tumor lesions (mean days ± SD) and % complete protection from tumor development for an observation period of 100 days was recorded. In experiments 1 and 2 (upper part) the effect of IL-12 given at different times after challenge was tested. IL-12 was given at 0.25 µg per day i.p. for 4 days either on days 2-5, 5-9 or 12-15 following challenge. In experiments 3 and 4 (lower part) the effect of antibody-mediated depletion of lymphocyte subsets on vaccination followed by IL-12 treatment given days 2-5 following challenge was tested. Mice were depleted of $CD4^+$ or $CD8^+$ T cells or both or of NK cells by injecting the appropriate antibodies on days -4, -1, +1, +4 and +8 with regard to tumor challenge. n.t. - not tested

Table 5

Vaccine	IL-12	1st challenge	2nd challenge	mean tumor free interval % complete protection (n)*
Vp53-wt	+	GL261	GL261	>50 100% (9)
VRG	+	GL261	GL261	>50 100% (8)
None	-	-	GL261	18.3. ± 3.2 / 0% (8)
Vp53-wt	-	GL261	B16.F10	23.3 ± 2.4 / 22% (9)
Vp53-wt	+	GL261	B16.F10	28.3 ± 4.0 / 11% (9)
VRG	-	GL261	B16.F10	22.7 ± 2.3 0% (3)
None	-	-	B16.F10	13.7 ±2.6 0% (9)

Groups of mice were vaccinated with 2 x 10^7 pfu of the Vp53-wt or VRG vaccine. They were challenged 14 days later with 1 TD_{100} of GL261 cells. Some of the groups were treated with IL-12 at 0.25 µg/day i.p. on days 2-5 following challenge. Mice that remained tumor-free were rechallenged with 10 TD_{100} of GL261 cells or B16.F10 cells. Additional naive mice were also challenged at this time. Data show the mean tumor-free interval after the 2nd challenge in days ± SD and the % of mice that did not develop tumors over the 100 day observation period after the 2nd challenge. n - number of mice used for the 2nd challenge.

Figure 2



Legend: Mice were injected with GL261 cells s.c. Mice with clearly visible tumors (0.3 - 0.5 mm in diameter) were vaccinated with 2 x 107 pfu of the Vp53-wt or the VRG vaccine. The following day IL-12 treatment was initiated. The graphs show the summary of 3 experiments. In the first 2 experiments mice developed tumors rapidly and the experiments were terminated on day 35. In the 3rd experiment tumors developed more slowly and mice were observed for 70 days. Data are expressed as % mice of all 3 experiments that had small to medium size tumors (⊞), large tumors requiring euthanasia (□), or had no longer any evidence of tumors (■).

A combination of IL-12 and Vp53-wt causes regression of already established tumors: Cancer patients are not treated prophylactically but rather after the cancer is sizable enough for diagnosis. Immunotherapy is generally initiated after debulking of the tumor by surgery where possible, followed by conventional therapy. To test if the Vp53-wt vaccine in combination with IL-12 induced an immune response that could eliminate small existing tumors, groups of C57Bl/6 mice were first inoculated with 1 TD_{100} of GL261 cells. Once the tumors became visible (0.3-0.5 mm in diameter) the mice were vaccinated either with the Vp53-wt vaccine or with the VRG construct. The following day IL-12 treatment was initiated for 5 days at 0.25 μg/mouse. As shown in Figure 2, the combination treatment of IL-12 and Vp53-wt vaccine resulted in complete regression of subcutaneous GL261 tumors in over 40% of mice; 2 of these 20 mice had a tumor recurrence later on, the others remained disease-free. IL-12 in combination with the control vaccine had a temporarily inhibitory effect on tumor growth and caused complete regression in only 20% of mice.

DNA tumor vaccines to p53: We generated a number of DNA vaccines expressing either full-length or truncated (removal of base pairs 1-70, i.e., transactivation domain) wild-type or mutant p53 (using the same mutations that were used for construction of vaccinia virus recombinants, i.e., single point mutation in position 135 and double point mutation in positions 168 and 234) and tested them for induction of protection against a subclone of a MethA tumor cell line expressing the same double mutation of p53 (135/268) as one of the DNA vaccines. Results were variable. In the experiment shown in Table 6, up to 70% complete protection could be achieved against the MethA-34 tumor cell line regardless of the form of p53 (wild-type or mutant) or the promoter driving expression of p53 (SV40 or CMV). In these experiments, mice that were completely protected remained resistant to further challenge with a 5 fold higher dose of the same tumor cell line while they remained susceptible to challenge with a high dose of an unrelated tumor cell line (i.e., CT-26) or a $p53^-$ tumor cell line (i.e., t(10)1rasE7E6) (Table 7).

Table 6

Vaccine	Complete Protection
pVR1012.2	2 / 10
pVR1012.2p53mu168/234	7 / 10
pVR1012.2Dp53mu168/234	6 / 10
pSV2p53mu168/234	6 / 10
pSV2p53wt	6 / 10

Mice were immunized i.m. with 100 μg of DNA. They were challenged 3 months later with MethA-13 cells and tumor development was recorded.

Table 7

Mice	Challenge	Complete Protection
DNA vaccine + MethA-34	MethA-34	8 / 8
Naive	MethA-34	0 / 5
DNA vaccine + MethA-34	CT-26	0 / 9
Naive	CT-26	0 / 5
DNA vaccine + MethA-34	t(10)1rasE7E6	0 / 8
Naive	t(10)1rasE7E6	0 / 5

This experiment is a continuation of the one shown in Table 6. Mice that were immunized with the p53 expressing DNA vaccines and remained tumor-free after the 1st challenge were rechallenged with 10 TD_{100} of different tumor cells. Tumor development was recorded.

In an additional experiment, the DNA vaccine based on the pVR1012 vector expressing the 168/234 double mutation of p53 showed partial protection in C57Bl/6 mice against challenge with GL261 cells (Table 8). Significant protection could not be induced against other tumor cell lines such as the adenocarcinoma line 66.1 or the CT-26 colorectal carcinoma line, indicating that further modifications are needed to improve the efficacy of the DNA vaccine.

Table 8

Vaccine	% Complete protection	Tumor free interval (mean days ± SD)
pVR1012.2	10	27.4 ±10.8
pVR1012p53mu168.234	30	32.9 ±10.4
pSV2p53mu135	11	27.2 ± 13.0
pSV2p53mu168.234	20	27.4 ± 8.5

Legend: Groups of 9-20 mice were injected with 50 µg of the DNA vaccine. They were challenged with 1 TD_{100} of GL261 cells. Data represent the percent of mice that remained completely tumor-free during the 50 day observation period as well as the mean tumor free interval after challenge (days ±SD).

As already mentioned, the level of protection achieved with the DNA vaccine was highly variable and altogether not satisfactory. Reasoning that the nuclear localization domain of p53

might render this protein somewhat inaccessible for association with MHC determinants, a pre-requisite for activation of T cells, we incorporated the following changes into the pVR1012 based DNA vaccine expressing a biologically inactive form of the p53 protein. First we added a leader sequence derived from adenovirus. This construct provided clearly superior protection to challenge with MethA cells compared to the original DNA vaccine (Table 9). We then added a ubiquitin sequence to improve association with MHC class I determinants. Testing of this vaccine is ongoing. As a third modification we are currently adding a leader sequence and an l-amp sequence to facilitate association with MHC class II determinants. Construction of this vaccine has not yet been completed.

Table 9

Vaccine	Complete Protection
pVR1012.2	5 / 18
pVR1012.2p53mu168/234	4 / 16
pVR1012.2p53mu388-leader	11 / 14

Mice were immunized twice i.m. with 50 μg of DNA. They were challenged 1 month after the 2nd dose with MethA-13 cells and tumor development was recorded. The pVR1012.2p53mu388-leader vaccine expresses a biologically inactive form of p53 with a point mutation in the tetrameization domain as a fusion protein with an adenoviral leader sequence. In vitro experiments showed that p53 encoded by this construct accumulates in the ER.

(7) CONCLUSIONS

Our data shows that vaccines expressing wild-type p53 can induce protective immunity reflected by complete resistance or delayed onset of tumor lesions to challenge with tumor cells expressing high levels of either wild-type or mutant p53. The efficacy could be increased by using IL-12 in combination with the vaccine. This regimen could induce complete regression of pre-existing tumors in ~ 45% of mice. Mice that were protected against the initial low-dose challenge remained completely resistant to an additional challenge with a higher dose of the same tumor cells. A number of immune effector mechanisms contribute to protection, most notably $CD4^+$ and $CD8^+$ T cells as well as NK cells. Upon vaccination with the vaccinia virus recombinant Th2 cells played a predominant role in tumor rejection. Different tumor cells showed distinct susceptibility to the p53-induced immune effector mechanisms; while the GL261 cell line was highly susceptible, others such as the B16.F10 melanoma cell line were resistant. We have not yet established which host or tumor cell parameters determine the efficacy of the p53 vaccine. We did not observe a correlation between susceptibility and levels of p53 expression (the susceptible GL261 cell line expresses less p53 than the more resistant 66.1 mammary adenocarcinoma cell line) or levels of TGF-ß secretion or the H-2 haplotype (both the susceptible GL261 and the resistant B16.F10 cell lines are of C57Bl/6 origin). Other parameters such as: levels of MHC class I expression, secretion of IL-10, and susceptibility to T cell-mediated cytolysis are being investigated. We are also testing

if we can improve the efficacy of the Vp53-wt vaccine to the more resistant tumors by using a combination treatment with recombinant mouse IL-12.

The efficacy of the p53 expressing DNA vaccines was initially disappointing. We hypothesize that p53 as expressed by a plasmid vector is mainly targeted to the nucleus and thus not available for presentation with MHC determinants. We reconstructed the DNA vaccine by cloning a fusion gene composed of the signal sequence of adenovirus and a mutant p53 (mutated in position 338 to generate a biologically inactive form unable to form heterodimers). The modification clearly improved the efficacy of the DNA vaccine.

These experiments will continue through a recently funded grant from the National Cancer Institute (1RO1 CA78492-01), entitled "Cancer Vaccine to p53." The start date of the NCI grant has been delayed until the day after the funding for our current DAMD grant is terminated.

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(9)APPENDICES:

Manuscript submitted:

Blaszczyk-Thurin, M., Deng, H., Wysocka, M., Xiang, Z., Jost, M., Rodeck, U., and Ertl, H.C.J. 1998. A recombinant vaccine to wild-type p53 for cancer prevention.

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FINAL REPORT INFORMATION SUMMARY

Abstract from presentation at the DOD/USAMRMC Breast Cancer Research Program:
An Era of Hope
October 3-November 4, 1997

VACCINES BASED ON p53 PROVIDE PROTECTION TO TUMOR CHALLENGE IN AN ANIMAL MODEL

The goal of our research is to test vaccines to p53 for the induction of protective immunity to tumor cells overexpressing wild-type p53 or expressing mutated p53. p53 is a tumor suppresser protein that controls cell growth. Mutations of p53, which cluster in well-defined hot spots of the gene, generally results in overexpression of the p53 protein due to an extension of its half life. P53 mutations are the most commonly found abnormality in human cancer. Up to 90% of human breast cancers are due to p53 mutations. Mutated, as well as overexpressed, proteins can potentially serve as target antigens for immuno-surveillance. Tumor cells carrying such proteins commonly fail to directly induce an immune response that is sufficiently efficacious to cause their elimination. Nevertheless, immune responses induced by vaccines can recognize tumor cells that carry the appropriate antigen and thus limit their spread. Within the realm of this application we have constructed a number of different vaccine prototypes, based on vaccinia virus recombinants and plasmid vectors. These vaccines either express wild-type p53 or two different forms of mutant p53, with one set of constructs carrying a mutation of the amino acid at position 135 and a second carrying a double mutation - one at 234 and a second one at 168. A number of tumor cells were analyzed for p53 expression by amplification of transcripts encoding the mutational hot spot region of p53 by RT-PCR. The PCR products were sequenced to identify p53 mutations. Most of the mouse tumor cell lines carried wild-type p53; one MethA induced tumor line gave variable results for the p53 sequence, upon subcloning a line was isolated which contained p53 mutations at 168 and 234. This line was expanded and used for further studies. Tumor cells were initially titrated in syngeneic mice to determine a dose that caused tumors within 21 days in all of the animals. Most of the vaccine studies to date have been conducted with the GL261 cell line which expresses wild-type p53 and the MethA tumor cell line which expresses a double mutation.

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A recombinant vaccine to wild-type p53 for cancer prevention[1]

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Running Title: A cancer vaccine to p53

Key Words: vaccine, cancer, p53, immune effector mechanisms, cryptic epitopes

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[2]H. Deng is the recipient of a fellowship from the Taxin Foundation.

SUMMARY

A vaccinia virus recombinant expressing mouse wild-type p53 (Vp53-wt) was shown to induce partial protection in mice against challenge with tumor cell lines expressing high levels of endogenous wild-type p53. On average, 50% of Vp53-wt-vaccinated C57Bl/6 mice remained disease-free upon subcutaneous challenge with a GL261 glioma cells while ~30% of Balb/c mice were completely protected against challenge with the 66.1 breast cancer cell line. Most mice that developed cancer in spite of vaccination showed a delayed onset of tumor growth. Protection against GL261 cells was mediated by T cells of both subsets (i.e., CD4 and CD8) as well as natural killer (NK) cells. Interestingly, IL-4, which is indicative of a Th2 type immune response, was essential in the GL261 tumor model. In contrast, the Th1 cytokine interferon (IFN)-g was not required. As tested in the GL261 model, Vp53-wt-vaccinated mice that remained disease-free upon challenge with a minimal tumorigenic dose of cells were then completely resistant to further challenge with increased doses of the same tumor cells. Vp53-wt vaccinated mice did not develop overt symptoms of autoimmunity; all mice remained healthy and showed no evidence of impaired liver, kidney or bone marrow function. In summary, a vaccine based on a self protein, i.e. p53, was shown to protect against p53 overexpressing tumors indicating that the induced immune effector mechanisms selectively recognized p53 overexpressing tumors without targeting normal cell with low levels of nuclear p53.

INTRODUCTION

Vaccination to infectious agents is based on the administration of antigens that are foreign to the immune system of the host. Depending on the type and form of the antigen used, both arms of the specific immune system, i.e., B and T lymphocytes, are activated leading to the development of memory cells. Upon re-encounter with the same pathogen, memory cells rapidly turn into activated effector cells that are capable of warding off an overwhelming infection. In contrast to infectious agents, cancer cells show only subtle antigenic differences when compared to normal cells. Many of these differences, such as deletions that result in lack of protein expression, can not be recognized by the immune system. Others, such as changes in glycosylation, are unsuited to induce a T cell response, which is best equipped to eliminate tumor cells. Point mutations found in tumor suppressing proteins such as p53 (Hollstein et al., 1991; Nigro et al., 1989) can provide T cell targets if presented in the context of the appropriate major histocompatibility complex (MHC) determinants. Nevertheless, T cell recognition of individual epitopes is ruled by Ir-gene control (Benacerraf and McDevitt, 1972), thus limiting the usefulness of single epitope vaccines to a fraction of an outbred population. In contrast to the limited qualitative antigenic differences between normal and tumor cells, quantitative differences are frequent and provide potential targets for immunotherapeutic and vaccination strategies. A self protein overexpressed by malignant cells, such as mutated or wild-type p53 (Yanuck et al., 1993; Noguchi et al, 1994; Roth et al, 1996; Xu et al, 1994), can trigger a T cell-mediated immune response by exposing so-called cryptic epitopes (Lanzavecchia, 1994; Dalton et al, 1993) which, when present at lower, physiological levels, have too low of an avidity to MHC determinants to reach the threshold needed for T cell tolerization or activation. However, inducing a response to cryptic self epitopes raises the possibility of adverse autoimmune reactions.

In this study we tested the efficacy of a recombinant vaccinia virus vaccine expressing high levels of unmodified mouse p53. The p53 protein is frequently overexpressed in many of the most common types of human cancer (Hollstein et al, 1991; Nigro et al, 1989). Wild-type p53 acts by

curtailing cell cycle progression and by inducing apoptotic cell death upon severe DNA damage. Mutations which cluster in well-defined regions of the *p53* gene cause structural changes in the p53 protein leading to its functional inactivation. Most *p53* mutations significantly prolong the half-life of the protein leading to overexpression of nonfunctional p53. Tumors that carry wild-type *p53* also frequently overexpress p53 (Xu et al., 1994). The p53 protein thus provides a good model to test whether the immune response to self epitopes can cause tumor rejection without inducing overt symptoms of autoimmunity.

Our results show that the vaccinia virus recombinant expressing full-length mouse wild-type p53 induces protective T and NK cell-mediated immunity to transplantable mouse tumor cell lines expressing spontaneously high levels of wild-type p53.

MATERIALS AND METHODS

Mice: Female C57Bl/6, Balb/c, and mice defective in IFN-g production, GKO (knock-out) (Dalton et al., 1993), were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained at the Animal Facility of The Wistar Institute. CD4KO mice (Xiang et al., 1995), lacking $CD4^+$ T cells, ß2m KO mice (Zijlstra et al., 1990), lacking ß2-microglobin and, thus, MHC class I molecules and $CD8^+$ T cells, perforin-KO (PKO) mice (Walsh et al., 1994), that are unable to use the perforin pathway for lymphocyte-mediated cytolysis, µMT mice (Kitamura and Rajewsky, 1991), that due to a defect in the Ig transmembrane domain lack mature B cells, IL-4KO mice (Kuhn et al., 1991), which are unable to produce interleukin (IL)-4, and severe combined immunodeficient (SCID) mice were bred at The Wistar Institute's Animal Facility. All of the knock-out mouse strains except for the CD4KO strain were backcrossed >5 times onto the C57Bl/6 background.

Cells: GL261, a murine glioma cell line of C57Bl/6 origin, was obtained from The National Cancer Institute (Frederick, MD). The 66.1 mammary adenocarcinoma cell line was kindly

provided by Dr. Amy Fulton, University of Maryland, Baltimore, MD. C57Bl/6 fibroblasts were established from C57Bl/6 embryos at 16-18 days of gestation. The (10)1 line is a Balb/c fibroblast line with a spontaneous loss mutation of both p53 alleles (Harvey and Levine, 1991). The t(10)1ras.E7E6 tumor cell line was generated by stable transfection of (10)1 cells using a triple promoter vector (Overell et al., 1988) expressing v-Harvey-ras under the control of the long terminal repeat of Rous sarcoma virus, the E7 protein of human papilloma virus (HPV)-16 under the herpes simplex virus thymidine kinase promoter and the E6 protein of HPV-16 under the SV40 promoter. Cells were grown *in vitro* in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics.

Expression of p53: Amplicons obtained by reverse transcriptase (RT)-polymerase chain reaction (PCR) of *p53* mRNA of tumor cells were sequenced at the Nucleic Acid Facility of The Wistar Institute using a dideoxynucleotide termination reaction. Expression of p53 protein was determined by immunostaining of cell monolayers with the p53-specific monoclonal antibody (mAb) Ab-1 (Oncogene Inc., Cambridge, MA) followed with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin (Ig). Counterstaining of nuclear DNA was performed with propidium iodide. Cells were analyzed by confocal microscopy at the Morphology Core Facility of The Wistar Institute.

Expression of MHC class I determinants: Cells were tested for MHC class I expression by incubating 2×10^5 cells with 20 µl of hybridoma culture supernatant containing the mAb SF1-1.1.1 (ATCC, Rockville, MD) with specificity for K^d or the mAb 28-14-8S (most kindly provided by Dr. E. Heber-Katz, The Wistar Institute) which reacts with both D^b and L^d for 45 minutes on ice followed by 30 minutes of incubation with an FITC-labeled goat anti-mouse Ig antibody. Cells were subsequently analyzed in a fluorescent activated cell sorter.

Secretion of cytokines: The 24 hr supernatants of tumor cells ($2x10^5$/ml) were assayed for the active and latent form of TGF-ß (Bellone et al., 1997) and for IL-3/GM-CSF (Xiang et al.; 1995) using previously described biological assay systems.

Vaccinia virus recombinants: The Vp53-wt recombinant was constructed as follows: the DNA fragment encoding mouse full-length wild-type p53 was excised from the pGEM-p53 vector (kindly provided by Dr. Thanos D. Halazonetis, The Wistar Institute) (Waterman et al., 1995) and cloned into the pSC11 transfer vector for vaccinia virus (Charabarti et al., 1985). A recombinant vaccinia virus containing the *p53-* sequence was generated by homologous recombination with vaccinia virus strain Copenhagen using thymidine kinase-negative cells (TK^-) (Perkus et al., 1989). The recombinant virus was tested by PCR for the presence of the *p53* sequence. Expression of p53 protein was analyzed by Western blot analysis (Towbin et al., 1979) of p53 negative (10)1 fibroblasts infected with the vaccinia virus recombinant using commercially available mAbs to p53 (Ab-1 and Ab-3). The vaccinia rabies virus glycoprotein (VRG) recombinant, which is also based on the Copenhagen strain of vaccinia virus (Wiktor et al., 1991), was used as a negative control throughout the experiments. Vaccinia virus recombinants were purified by high speed centrifugation onto a 36% sucrose cushion and titrated on HeLa or thymidine kinase deficient cells as described (Wiktor et al., 1984).

Immunization and challenge of mice: Tumor cell lines were titrated in syngeneic mice by s.c. inoculation to establish the tumorigenic dose (TD_{100}) for each cell line, defined as the number of cells required for tumor formation in 100% of unvaccinated animals within ~15 to 30 days after inoculation. Protection studies were carried out by injecting 6 to 12 week-old mice subcutaneously (s.c.) with a single dose of 2×10^7 plaque forming units (pfu) of Vp53-wt virus or VRG virus in 200 µl of saline. Mice were challenged 2 or 3 weeks later s.c. with tumor cells. Animals were monitored three times per week for development of tumors, and tumor size was estimated. Mice

which developed tumors >1.5 cm in diameter or showed signs of functional impairment due to the tumor mass were euthanized according to Institutional Animal Care and Use Committee guidelines. Some of the mice that remained tumor-free after the initial challenge were rechallenged 3-5 months later with 1 - 5 x10^6 GL261 cell given s.c.

Antibody depletion studies: *In vivo* depletion of specific T cell subsets was accomplished by injecting mice intraperitoneally (i.p.) with rat mAbs specific for mouse CD4 (i.e., GK1.5, ATCC) or CD8 (i.e., 53.6.7, ATCC), using 0.2 ml aliquots of a 1:10 dilution of antibody derived from the ascitic fluid of SCID mice injected with hybridoma cells. The antibodies were injected on days -4, -1, +1, +4 and +8 relative to tumor cell injection (day 0). Preliminary experiments had shown that this treatment resulted in >95% reduction of the appropriate T cell subset. Deletion of natural killer (NK) cells was achieved using a rabbit serum against asialo-GM1 ganglioside (Wako Inc., TX) at the dose recommended by the manufacturer. Control mice were inoculated with the same dose of a rat IgG immunoglobulin (Sigma Chemical Co., St. Louis, MO.). Depletion of cytokines was achieved by injecting mice 5 times (days -4, -1, +1, +4, +8) with 100 μg per dose of rat mAb specific for IFN-γ (XMG-1, ATCC) or IL-4 (11-B-11, ATCC) which were purified using a fast-flow protein G column (Pharmacia Biotech, Uppsala, Sweden).

Immunohistochemistry: Frozen sections were stained using the avidin-biotin-peroxidase Vectastain Elite ABC kit (Vector Laboratories Burlingame, CA). Sections were developed with 3',3'-diaminobenzidine tetrahydrochloride (Sigma St. Louis, MO.) and counterstained with 1% hematoxilin (HE). Sections were analyzed in a SONY up-5500/5600 microscope with the computational ability to count the number of stained cells per section and to determine the percentage of peroxidase stained area/total section area.

RESULTS

Characterization of tumor cell lines: Initially some of the basic characteristics of the tumor cell lines used in this study were defined. The p53 gene was analyzed for potential mutations and the p53 protein was tested for levels of expression. Both tumor cell lines, i.e., GL261 and 66.1, lack p53 point mutations (Table 1) but express abnormally high levels of nuclear p53 protein when compared to embryonal fibroblasts (Figure 1, Table 1) as does the B16F10 melanoma cell line used as a control for some of the in vivo experiments (not shown). The t(10)1rasE7E6 cell line, which is a transformed fibroblast cell line with a spontaneous loss of both p53 alleles, was used as a negative control. Both the GL261 and the 66.1 cell line expressed readily detectable levels of MHC class I determinants that were upregulated upon pre-treatment of tumor cells with IFN-γ (data not shown). The t(10)1rasE7E6 cell line expressed K^d while L^d could not be detected (data not shown). All of the cell lines secreted TGF-ß, which was predominantly (>90%) in the latent, biologically inactive form; whereas the mammary carcinoma cell line 66.1 and the transformed fibroblast cell line secreted IL-3/GM-CSF at significant amounts. The GL261 cell line showed the lowest tumorigenicity in syngeneic mice. Upon subcutaneous inoculation, 10^5 Gl261 cells as compared to $4x10^4$ 66.1 cells and $2x10^4$ t(10)1rasE7E6 cells were required to form tumors in 100% of inoculated mice.

The Vp53-wt vaccine induces protective immunity to tumor challenge. To test the efficacy of the vaccinia virus recombinant expressing mouse wild-type p53, mice were immunized s.c. with a single dose of 2×10^7 pfu of virus. Control mice injected with the same dose of a vaccinia virus recombinant expressing an unrelated viral antigen (Wiktor et al., 1984) were used to distinguish anti-tumor effects caused by specific immune responses to p53 from non-specific bystander effects due to a reaction to the vaccine carrier. Mice were challenged with 1 TD_{100} of tumor cells 2 weeks after immunization. As shown in Table 2, mice immunized with the Vp53-wt vaccine were partially

protected (i.e., either complete protection, or delayed onset of tumor growth) against challenge with $p53^+$ tumor cells. Table 2 shows that complete tumor rejection was achieved in 50 % of immunized C57Bl/6 mice challenged with GL261 cells, while only 30% of Balb/c mice challenged with 66.1 cells remained disease-free. Most of the mice that developed tumors in spite of p53 vaccination showed a delayed onset of tumor development that was statistically significant. The control VRG control vaccine reproducibly caused some protection against the GL261 cell line (see Tables 2 and 3), which is presumably related to activation of innate immune responses. No protection was achieved against the $p53^-$ t(10)1rasE7E6 cell line. Vaccination with the Vp53-wt vaccine did not protect against challenge with a higher dose (10 TD_{100}) of any of the tumor cells tested. However, Vp53-wt vaccinated mice that remained tumor-free after the initial challenge with 1 TD_{100} GL261 cells were completely resistant to a subsequent challenge with 10-50 TD_{100} GL261 cells (Figure 2) which rapidly killed all of the control mice. Vaccinated and then GL261 challenged mice remained susceptible to challenge with an unrelated p53-overexpressing melanoma cell line (data not shown). These data indicate that the initial tumor cell challenge provided a booster effect in vaccinated mice leading to enhanced resistance to a further encounter with the same tumor cells.

Immune effector mechanisms that mediate tumor rejection: The immune mechanisms that cause tumor rejection in Vp53-wt vaccinated mice were defined using the GL261 tumor cell system. To study lymphocytic infiltrations of tumors in situ, GL261 tumors from VRG and Vp53-wt vaccinated mice were analyzed by immunohistochemistry. Tumors from 2-3 mice of ~ 1 cm in diameter were fixed and thin sections were stained for cell surface markers specific for T helper cells (CD4), cytolytic T cells (CD8), B cells (B220), granulocytes, macrophages (mac-1), and activated antigen presenting cells (APCs) (B7.2) using commercially available antisera or mAbs. Tumor sections showed three distinct areas: solid tissue which was fairly homogeneously infiltrated with mononuclear cells, necrotic areas with a less pronounced infiltrate, and an interphase between solid and necrotic parts of the tissue which showed intense infiltration with

mononuclear cells. The most striking observation was the increased content of $CD8^+$ T cells in solid tumor tissue of Vp53-wt vaccinated mice as compared to tumors from VRG immune control mice (Figure 3). By contrast, $CD4^+$ T cells already present at a high level in tumors of control mice only increased about 2-fold in these areas (Figure 3). Only a few T cells were scattered throughout the necrotic part of the tumors or at the interphase, where granulocytes, mac-1^+ cells, and $B220^+$ cells were more numerous. Cells expressing B7.2, a co-stimulatory molecule expressed on APCs such as dendritic cells, were found predominantly in the solid tissue of Vp53-wt vaccinated mice concomitant with $CD4^+$ and $CD8^+$ T cells (Figure 4). Taken together, these data indicate that additional accumulation of $CD4^+$ T cells and massive infiltration of tumor tissue with $CD8^+$ T cells are consequences of Vp53-wt vaccination associated with recruitment of APCs and inflammatory cells as well as B cells.

The functional contribution of different immune effector mechanisms in providing protection in Vp53-wt vaccinated mice to GL261 tumor cell challenge was assessed in knock-out mice with defined deficiencies in $CD4^+$ T helper cells (CD4KO), $CD8^+$ cytolytic T cells (ß2KO), B cells (μMt), perforin (PKO) or cytokines, i.e., IFN- γ (GKO) indicative for a Th1 type response and IL-4 (IL-4KO) reflecting a Th2 type immune response. Knock-out mice commonly develop alternative pathways to compensate for their defect, therefore, where possible, data obtained in knock-out mice were confirmed by antibody-mediated ablation of lymphocytes or cytokines in fully immunocompetent C57Bl/6 mice.

CD4KO mice, ß2KO, PKO and IL-4KO mice were not protected against challenge with the GL261 cell line upon vaccination with the Vp53-wt construct (Table 3). In contrast, tumor rejection was not impaired in μMT and GKO mice (Table 3). These data were confirmed by transfer of mAbs given just prior to and after tumor challenge; treatment with antibodies to CD4, CD8 or IL-4 abrogated protection while transfer of an mAb to IFN- γ had no effect (Table 4). In fact in 5 out of 5 experiments, treatment with an antibody to IFN- γ at the time of tumor challenge

slightly but reproducibly increased the level of complete protection. NK cells also appeared to be required to limit tumor growth in Vp53-wt vaccinated mice, as depletion of this lymphocyte subset by treatment with a rabbit serum to asialo-GM-1 also resulted in loss of protection (Table 4). Nevertheless, the caveat should be added that this marker is also expressed on a subpopulation of T cells. Furthermore, mice lacking $CD4^+$ T cells or IL-4, either due to a genetic defect or upon treatment with the appropriate mAb, showed a strikingly accelerated growth of tumors; this was not observed in mice lacking any of the other immune effector mechanisms (Figure 5).

To test which immune effector mechanisms contributed to the augmented protection upon Vp53-wt vaccination followed by challenge with a moderate dose of GL261 cells (Figure 1), mice were rechallenged with a 10 fold higher dose of GL261 cells and at the time of rechallenge treated with depleting doses of anti-CD4 or anti-CD8 antibodies. As shown in Table 5, depletion of either T helper cells or cytolytic T cells only partially affected tumor rejection in these mice. These data again stress that the initial tumor cell challenge had very strongly boosted the immune response so that depletion of one T cell subset did not abolish the organism's ability to successfully cope with a fairly large dose of tumor cells.

Taken together, analysis of the immune effector mechanisms indicates that: (i) activation of innate immune effector mechanisms, i.e., NK cells, and T helper and cytolytic T cells were required, (ii) protection required IL-4, a cytokine associated with activation of Th2 type T cells while IFN- γ, a Th1 cytokine known to promote expansion of cytolytic T cells and expression of MHC determinants, was not required for protection in V53-wt vaccinated mice, (iii) cytolytic cells seem to utilize the perforin pathway for elimination of tumor cells, (iv) antibodies which can not readily bind nuclear proteins such as p53 play no role in rejecting GL261 cells, and (v) the immune response of vaccinated mice that survived the initial tumor cell challenge is of sufficient potency to reject tumor cells even in absence of one of the T cell subsets.

DISCUSSION

The most salient findings of this study can be summarized as follows: (i) vaccination with a vaccinia virus recombinant expressing mouse wild-type p53 induces partial protection in mice against a subsequent challenge with tumor cells expressing high levels of endogenous p53, (ii) protection depends on NK and T-cell mediated immune responses with Th2 type immune responses being of particular importance, (iii) the immune response upon vaccination with the Vp53-wt vaccine can be augmented by challenge with tumor cells, and (iv) the immune response induced by the wild-type p53 vaccine is apparently restricted to p53 overexpressing tumor cells as no overt symptoms of autoimmunity were observed in immunized mice.

Previous studies demonstrated induction of protective immunity to tumor challenge in mice using vaccines expressing either mutant or wild-type mouse or human p53 (Yanuck et al., 1993; Noguchi et al., 1994; Roth et al., 1996; Ropke et al., 1996). Although these earlier studies generally support the utility of p53 as a tumor rejection antigen, the model systems used were poorly suited to evaluate efficacy and practicality of p53 vaccination in humans. In one study foreign, i.e. human, p53 was used to vaccinate mice and the vaccinated mice were challenged with syngeneic tumor cells in which human p53 was constitutively expressed at high levels through transfection with a plasmid vector (Roth et al., 1996). It is debatable whether constitutive, forced expression of high p53 levels in transfected cells faithfully recapitulates deregulated expression of endogenous p53 in tumor cells; furthermore it is unclear whether protection in this system was related to the use of a foreign (i.e. human) antigen. Other studies used mutant forms of p53 and achieved protection against tumor cells expressing the homologous p53 mutation (Yanuck et al., 1993; Noguchi, Y. et al., 1994). T cell responses can be induced by a given p53 point mutation provided the affected amino acid is flanked by anchoring residues for the MHC determinants expressed by the tumor cells; if the mutation lies outside such a sequence it remains immunologically silent rendering immunization approaches utilizing vaccines expressing a mutant p53 sequence, such as synthetic peptides, ineffective. Thus, each of the known p53 mutants is

expected to be recognized and responded to by only a fraction of the individuals of an outbred human population, a phenomenon referred to as Ir-gene control (Benacerraf and McDevitt, 1972). This circumstance severely restricts the use of vaccines based on specific p53 mutations. In contrast to these earlier studies, we tested whether full-length wild-type mouse p53 could induce an effective immune response in mice to two different, syngeneic tumor cell lines expressing wild-type endogenous p53. Importantly, the tumor cells spontaneously express high levels of wild-type p53 rather than upon transfection providing a cellular target to the immune system that is similar to the target cells encountered in the tumor patient. Furthermore, as outlined in the preceding paragraph, the full-length p53 protein can potentially provide a broad spectrum of potential T cell epitopes for immune recognition.

It has been suggested that T cell recognition of self antigens expressed at abnormally high levels, such as p53 in tumor cells, is directed to so-called cryptic or subdominant epitopes (Sercarz et al., 1993; Lanzavecchia, 1994). The p53 protein is likely to contain cryptic epitopes as it is expressed at low, barely detectable levels in normal cells. Expression of p53 by the Vp53-wt vaccine is expected to cause synthesis of sufficient amounts of low-affine p53 epitopes for their association with MHC determinants resulting in the activation of T cells able to cause rejection of p53 overexpressing tumors as shown by the results presented in this manuscript.

Although vaccines based on overexpression of a self protein may induce T cell-mediated immunity to tumor cells, they potentially carry the risk of "horror autotoxicus", i.e., the induction of T cells that recognize and destroy normal cells. No overt signs of autoimmune reactions or disease was observed in Vp53-wt vaccinated mice; limited studies testing blood smears for a decrease in hematopoetic cells, serum for elevated liver enzymes or substances such as creatinin indicative of kidney dysfunction within weeks or several months after Vp53-wt vaccination failed to demonstrate any abnormalities (data not shown). The induction of tumor-specific immunity without adverse self-reactivity indicates that the mouse immune system is able to discriminate at the effector phase between cells expressing p53 at low (normal) and high (tumor cell) levels.

Upon immunization with the Vp53-wt vaccine, both $CD4^+$ T helper cells and $CD8^+$ cytolytic T cells were required for limiting tumor growth. Innate immune effector mechanisms such as NK cells were also essential. Cytolytic T cells to p53, once induced by the appropriate APCs presenting the epitope in context with MHC class I determinants as a first signal together with the required second signal in the form of co-stimulatory molecules (Linsley et al., 1991), may eliminate tumor cells expressing MHC class I bound cryptic epitopes of p53 by direct cytolysis. NK cells can directly kill target cells including those tumor cells that express no or only low levels of classical MHC class I determinants. $CD4^+$ T cells to p53 may be induced by Vp53-wt infected APCs or upon reprocessing of antigen released by cells killed upon infection with the cytopathic vaccinia virus recombinant. Production of IL-4 but not IFN- γ was essential for protection upon GL261 tumor cell challenge in Vp53-wt vaccinated mice. IL-4 production indicative of a Th2 type immune response that leads to the recruitment of inflammatory cells such as eosinophils whereas IFN-γ production is associated with a Th1 type immune response that sponsors activation of cytolytic T cells. Th1 type immune responses are generally considered to support tumor rejection (Tsung et al., 1997). However, the Th2 cytokine Il-4 has been shown to induce anti-tumor immunity in certain experimental models (Golumbek et al., 1997). This activity has been linked in part to improved antigen presentation (Cayeux et al., 1997) and in part to Th2 type immune effector mechanisms leading to tumor rejection associated with eosiniphilic infiltrates (Hock et al., 1993; Modesti et al., 1993; Pericle et al., 1994).

It is poorly understood how a given antigen initiates either a Th1 or Th2 type immune response. Low affine self epitopes may favor activation of Th2 over Th1 cells as remains to be investigated in more depth. Irrespective of their mode of action, $CD4^+$ T cells as well as IL-4 are apparently essential to check tumor cell growth in vaccinated as well as in control animals. In support of this conclusion, mice lacking either $CD4^+$ T cells or IL-4 due to antibody-mediated depletion or a genetic defect developed GL261 tumors more rapidly compared to control mice.

There was some variability from experiment to experiment with levels of complete protection in Vp53-wt vaccinated mice ranging from 40-60%. Vaccinated mice remained susceptible to challenge with a higher dose of tumor cells. This suggests that the immune effector cells were overwhelmed by the amount of tumor cells, resulting potentially in immunological exhaustion. Immune responses induced by tumor vaccines are not expected to achieve elimination of large tumor burdens but are more suitable for rejection of minimal residual disease following conventional therapy. In our system, mice that were protected upon vaccination with the Vp53-wt construct to such a limited dose of tumor cells were rendered completely resistant to subsequent challenge with a much higher dose of tumor cells, indicating that the first tumor cell challenge had boosted the immune response. Mice remained susceptible to challenge with other types of p53 overexpressing tumor cells suggesting that the booster effect was most likely not restricted to p53-specific T cells but had broadened to T cells that recognize distinct, as yet undefined, tumor-associated antigens. These data are encouraging as they suggest that a p53 vaccine-induced immune response not only establishes long-term memory but can also, upon encounter with residual tumor cells, broaden into a more effective anti-tumor immune response.

The Vp53-wt vaccine induces an immune response that protected 50%, on average, of C57Bl/6 mice challenged with minimal tumorigenic doses of the GL261 glioma line and significantly delayed tumor onset; vaccine efficacy to the breast cancer cell line was less impressive and mainly resulted in a delayed onset of tumor growth rather than in complete protection in the majority of mice. The characteristics of the tumor cells that correlate with susceptibility to the Vp53-wt vaccine-induced immune responses are currently unknown. We tested tumor cells for a number of parameters that might affect the efficacy of a specific immune response (Garrido et al, 1993; Chang et al., 1993) such as level of antigen, i.e., p53 expression, spontaneous and IFN-γ induced MHC class I expression, and secretion of the immunomodulatory cytokines TGF-ß and IL-3/GM-CSF. Both tumor cell lines overexpressed p53 and expression levels were slightly higher in 66.1 cells than in GL261 cells. Both tumor cell lines expressed readily detectable levels of MHC

class I determinants that could be augmented by treatment with IFN-γ. Both tumor cell lines secreted TGF-ß, a cytokine known to subvert the immune response to tumor cells (Chang et al., 1993). GM-CSF, which is known to recruit APCs thus facilitating induction of T cell responses, was secreted by the mammary carcinoma cell line only and apparently did not enhance the susceptibility of tumor cells to the Vp53-wt vaccine-induced immune response. In summary, none of these characteristics distinguished the susceptible GL261 cell line from the more resistant 66.1 cell line. The different levels of protection achieved with different tumor cell lines might be a consequence of genetic differences of the host strains, i.e., C57Bl/6 and Balb/c, used in the protection experiments. Nevertheless, in C57Bl/6 mice the vaccine induced good protection against the glioma cell line but was fairly inefficient in protecting against challenge with a wild-type p53 overexpressing melanoma cell line (results not shown). However, the GL261 cell line required a 5-fold higher dose of cell inoculum compared to the 66.1 cell line to cause tumor formation in control mice. This finding may in part reflect the higher immunogenicity of GL261 cells, which even in control mice formed tumors that contained a significant infiltrate of $CD4^+$ T cells. It is likely that immunogenic tumors are more susceptible to active immunotherapy. This hypothesis, which remains to be investigated further, is supported by data showing lack of protection in Vp53-wt vaccinated C57Bl/6 mice to challenge with a highly tumorigenic and poorly immunogenic melanoma cell line (results not shown).

In conclusion, we have shown that wild-type p53 presented by a vaccine virus recombinant can induce protective T cell-mediated immune responses to p53 overexpressing tumors without causing clinically overt autoimmunity. The underlying hypothesis, i.e., that the T cell response is directed to cryptic epitopes of the p53 protein that only reach the threshold needed for T cell recognition in transformed but not in normal cells, remains to be proven. In the near future high throughput microchip technologies will allow efficient identification of multiple overexpressed mRNA transcripts of self proteins in molecularly defined subgroups of tumors and in primary tumors obtained from patients. This development opens the prospect of combination vaccines expressing several of such proteins for the improved immunotherapy of cancer.

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FIGURE LEGENDS

Figure 1. The nuclear expression of p53 in murine tumor cells. Cell were grown on glass coverslips, immunostained for p53, and counterstained for DNA. Staining was assessed under a confocal microscope. The left panel shows immunofluorescence staining of p53, the right panel shows double staining for p53 and DNA.

GL261 (A, B), 66.1 (C,D), embryonal C57Bl/6 fibroblasts (E, F).

Figure 2. Mice vaccinated with the Vp53-wt vaccine and challenged with GL261 tumor cells are rendered completely resistant to further challenges with high doses of homologous tumor cells. Vp53-wt-immune mice that failed to develop tumors after the initial challenge were rechallenged with 1 x 10^6 or 5 x 10^6 GL261 cells (both ■) using naive C57B1/6 mice (⊞ 5 x 10^6 GL261 cells, □ 1 x 10^6 GL261 cells) for comparison. Mice were observed for 2 months and tumor development was recorded.

Figure 3. Immunohistochemical analysis of tumors from VRG or Vp53-wt vaccinated mice: Thin section of GL261 tumors from Vp53-wt or VRG immunized mice were stained with antibodies to cell surface markers. Sections (2 -3) of each tumor containing solid tissue (■), necrotic tissue (▨) or the interphase (▧) between the two were analyzed for % area of stained cells/ area of the section.

Figure 4. The kinetic of tumor development in mice depleted of lymphocytes: Groups of C57B1/6 mice were inoculated with 2 x 10^7 pfu of vp53-wt and one additional group was vaccinated with VRG virus (⊠). Fourteen days later the three groups of mice vaccinated with Vp53-wt virus were treated with monoclonal antibodies specific to CD4 (■) , to CD8 (□), or an antiserum to asialo-GM1 (⊞). Mice were then challenged with 2 X 10^5 GL261 cells and tumor size over time was recoded for individual mice. The graph shows representative results for a limited number of mice. Data for the complete experiment are shown in Table 4.

Table 1. Characteristics of tumor cells.

Tumor line	Tumor type/Origin	p53 Type[1]/Level[2]	1 TD_{100}[3]	TGF-ß secretion[4] act./total (pg/ml)	IL-3/GM-CSF[5] secretion
GL261	Glioma / C57Bl/6	wt/++	1×10^5	70/936	+/-
66.1	Mammary Carcinoma / Balb/c	wt/+++	4×10^4	27/759	+++
t(10)1rasE7E6	Transformed Balb/c fibroblasts	none	2×10^4	n.t.	+++

[1]The p53 mRNA was amplified by RT-PCR and the amplicon was sequenced, wt - wild-type. [2]Levels of nuclear p53 expression were determined by immunohistochemistry and by Western Blot analysis. The minimal tumorogenic dose (TD_{100}) of tumor cells was tested by subcutaneous inoculation of graded amounts of cells into syngeneic mice. Secretion of active and latent TGF-β[4] and of IL-3 and GM-CSF[5] was determined from 24 hrs tumor cell serum-free culture supernatants as described in the Material and Method section.

Table 2. The efficacy of the Vp53-wt vaccine upon challenge with different tumor cell lines.

Vaccine	Number of Mice	Cell Line	% Complete Protection	Tumor Free Interval (days ±SD)	Significance*
VRG	10	GL261	10	22.8±5.8	
Vp53-wt	10	GL261	50	39.0±12.7	0.0029
VRG	8	66.1	0	28.0±5.7	
Vp53-wt	8	66.1	30	45.3±9.1	0.017
VRG	9	t(10)1rasE7E6	0	15.4±1.6	0.038
Vp53-wt	9	t(10)1rasE7E6	0	18.9±2.9	

Groups of mice were immunized with 2 x 10^7 pfu of VRG or Vp53-wt virus. Two weeks later, mice were challenged with 1 TD_{100} of the different tumor cells. Mice that failed to develop tumors within the observation periods (50-100 days) were scored as being completely protected. For mice that developed tumors, the mean day from challenge until visible onset of tumor growth (>2 mm in diameter) ± standard deviation was recorded.

*Significance of the difference between onset in VRG and Vp53-wt vaccinated mice was calculated by a student t test.

The table shows the results representative of 2 to 3 experiments.

Table 3. Protection in mice with genetic defects in immune effector functions.

Vaccine	Number of Mice	Recipient	Defect	Tumor-Free Interval (days ± SD)	% Complete Protection
Experiment I					
VRG	10	CD4KO	T helper cells	26.0± 6.5	20
Vp53-wt	10	CD4KO	-	18.6± 3.1	20
VRG	10	ß2m	cytolytic T cells	25.5 ± 4.5	0
Vp53-wt	10	ß2m	-	26.8 ± 3.4	0
VRG	10	C57Bl/6	-	33.3±12.6	30
Vp53-wt	10	C57Bl/6	-	38.0± 9.6	60
Experiment II					
VRG	6	µMt	B cells	34.7±11.0	16
Vp53-wt	6	µMt	-	43.0±17.0	43
VRG	6	PKO	Cell-mediated cytolysis	27.9± 6.2	0
Vp53-wt	5	PKO	-	25.4± 8.1	0
VRG	8	C57Bl/6	-	35.0±11.0	12
Vp53-wt	9	C57Bl/6	-	41.0± 4.0	44

The table shows the results representative of 2 to 3 experiments. Mice were vaccinated with VRG or Vp53-wt. They were challenged 2 weeks later with GL261 tumor cells and tumor development was recorded. In each experiment control wild-type C57Bl/6 mice were tested in parallel.

Table 4. Protection in mice traeted with antibodies to lymphocyte subsets or cytokines.

Vaccine	Number of mice	Antibody Name	Antibody Specificity	Tumor-free Interval (days ±S.D.)	% Complete Protection
Experiment I					
VRG	10	-	-	33.2±12.6	30
Vp53-wt	10	-	-	33.0± 9.6	60
Vp53-wt	10	GK1.5	anti CD4	17.9± 3.4	0
Vp53-wt	10	53.6.7	anti CD8	25.1± 2.2	0
Vp53-wt	10	anti-GM1	anti NK	23.2± 2.5	10
Vp53-wt	6	XMG-1	anti IFN-γ	25.3±9.3	70
Experiment II					
VRG	6	-	-	31.7±5.1	16
Vp53-wt	6	-	-	33.0	66
Vp53-wt	6	11-B-11	anti-IL-4	42.6±12.0	16

The Table shows the results representative of 2 to 3 experiments each controled by C57Bl/6 mice vaccinated with the V53-wt or the control vaccine. Groups of C57Bl/6 mice were vaccinated with 2 x10^7 pfu of VRG or Vp53-wt virus. Two weeks later they were treated with antibodies specific for T-lymphocyte subsets or cytokines as detailed in the Material and Method Section. They were challenged with GL261 tumor cells and onset of visible tumors was recorded.

Table 5. The effect of T cell depletion on rechallenge of vaccinated survivors.

Treatment at Rechallenge	Number of mice	Tumor-free Interval (days ±S.D.)	% Complete Protection
None	8		100
GK1.5, anti-CD4	8	18.5±3.2	60
53.6.7, anti CD8	8	31.3±11.2	75

Mice were vaccinated with Vp53-wt virus and then challenged with 1 TD_{100} of GL261 cells. Mice from several experiments that remained tumor free for at least 100 days were pooled, treated with antibodies to CD4 or CD8 and then challenged with 10 TD_{100} of GL261 cells. Tumor development was recorded. The table shows the results representative of 2 experiments.

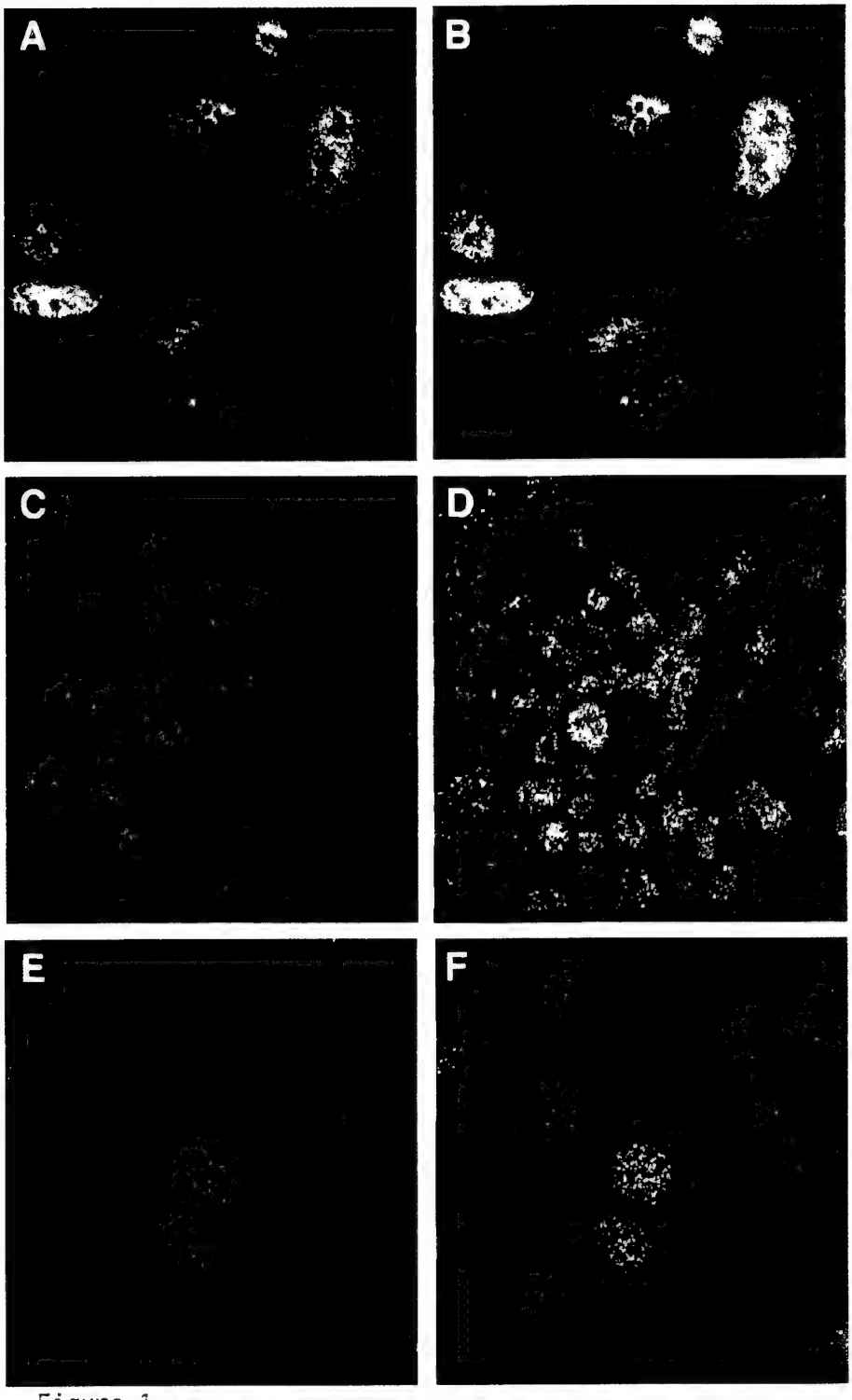

Figure 1

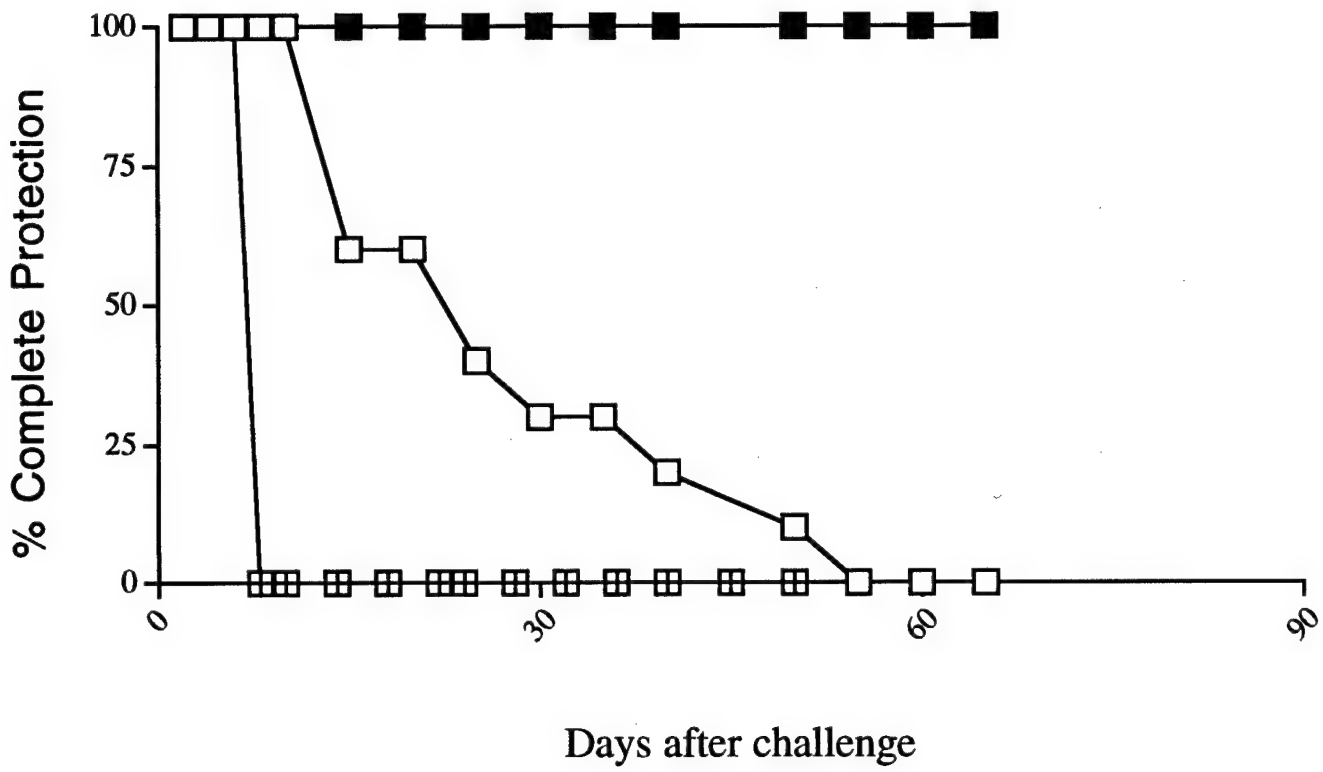
% Complete Protection
100
75
50
25
0
0
30
60
90
Days after challenge

Figure 2

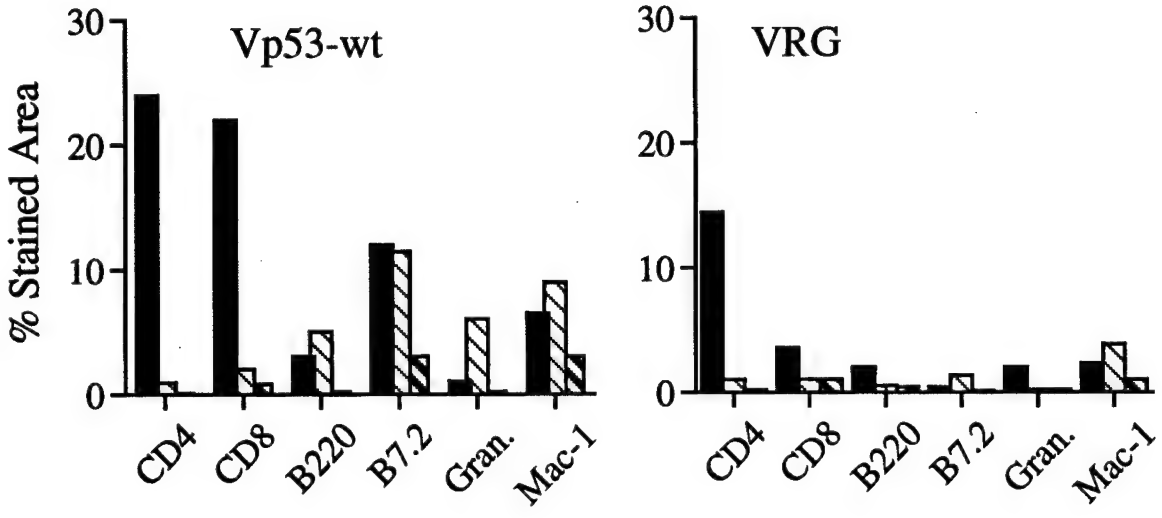
% Stained Area
30
20
10
0
Vp53-wt
CD4
CD8
B220
B7.2
Gran.
Mac-1
30
20
10
0
VRG
CD4
CD8
B220
B7.2
Gran.
Mac-1

Figure 3

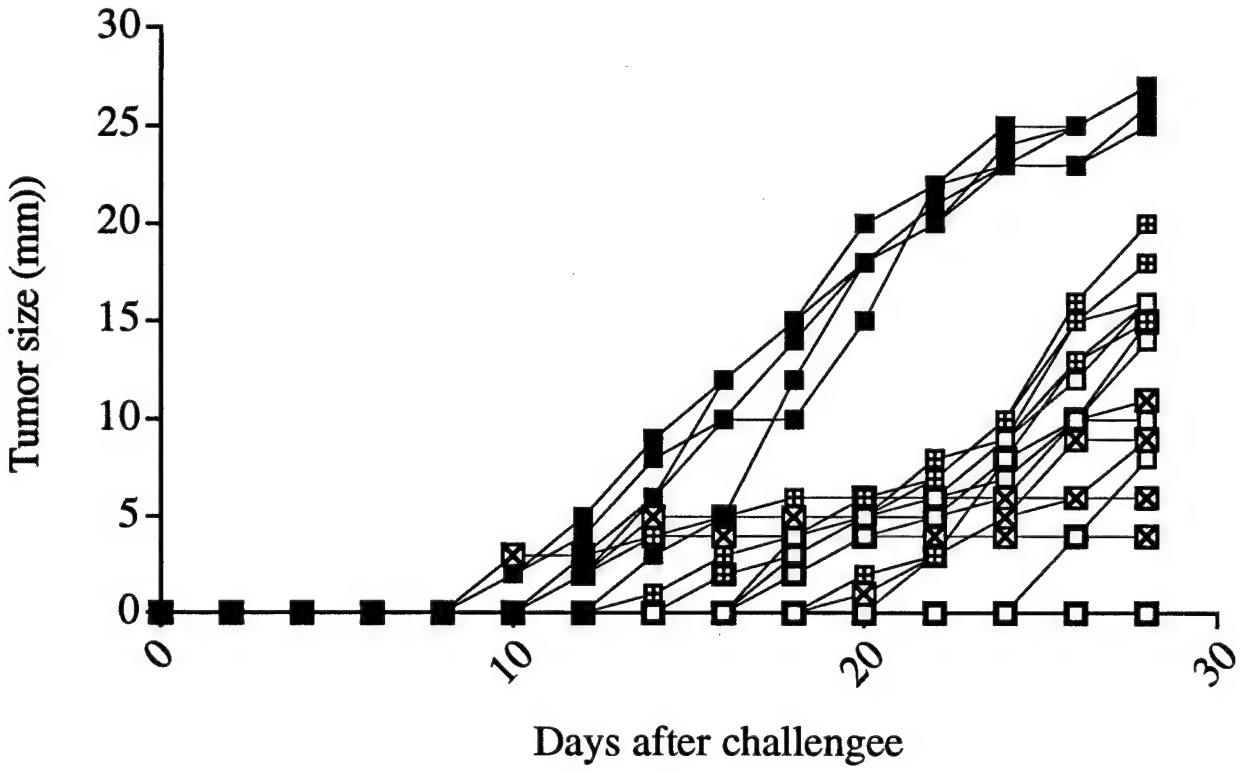

Figure 4

A monoclonal antibody to a multiphosphorylated, conformational epitope at the the carboxy-terminus of p53

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Key words: Enzyme-linked immunosorbent assay; Monoclonal antibody; p53; Peptide; Phosphorylation; Recognition

Mutations of the gene encoding the tumor suppressor protein p53 are the most common molecular alterations of cancer cells found in about half of all human tumors. Mutations which cluster in well-defined hot spots change the structure of the protein thus affecting its ability to bind to DNA. Post-translational modifications, primarily phosphorylation, might also influence how p53 binds to DNA or folds to its active tetrameric form. However, the lack of appropriate biochemical markers to characterize the status of phosphorylation in different cell types and in cells at different stages of tumor progression has prohibited such investigations. To generate a sensitive and phosphorylation-specific monoclonal antibody (mAb), we chemically synthesized the C-terminal 23 amino acid stretch of human p53 in a double phosphorylated form. The peptide 371-393, carrying phosphate groups on Ser378 and Ser392, was co-synthesized with a turn-inducing spacer and peptide 31D, an immunodominant T-helper cell epitope in mice of the H-2^k haplotype. After immunization and fusion of splenocytes with myeloma cells, a number of mAbs were obtained, from which mAb p53-18 emerged as a highly sensitive reagent. By enzyme-linked immunosorbent assay, p53-18, a mAb of the IgM isotype, recognized phosphorylated p53, expressed in insect cells infected with a recombinant baculovirus but not p53 expressed in *E. coli*. Moreover, murine p53 from insect cells could be immune purified with mAb p53-18. Mass spectrometry following tryptic digestion of the purified protein and liquid chromatography of the fragments verified the presence of phosphate groups at both Ser375 and Ser389. From the corresponding human protein fragments, mAb p53-18 bound to the immunizing peptide phosphorylated on Ser378 and on Ser392, but failed to cross-react with the unphosphorylated peptide, or peptides phosphorylated individually on either Ser378 or Ser392. The binding to the unphosphorylated peptide could be restored, however, if the peptide conformation was stabilized to that of an α-helix.

The immunogenic nature of the multiphosphorylated C-terminus of p53 is indicated by the finding that human sera, mostly from cancer patients, preferentially recognized the double phosphorylated peptide over the monophosphorylated or unphosphorylated analogs. Antibody p53-18 appears to be a highly useful biochemical marker to detect low levels of p53 protein in different tissues, and to be a key tool to characterize the phosphorylation status of the C-terminus of p53 protein originated from various sources.

Introduction

The tumor suppressor protein p53 is considered a guardian of the genome [1]. The p53 gene encodes a nuclear phosphoprotein that is altered by mutation or deletion in about 50% of human tumors [2]. p53 protein can be divided into five distinct regions. The transactivation domain covers the first 42 amino acid stretch. This region mediates the transcriptional activity of p53, which is directly correlated to its ability to suppress cell growth [3]. The next domain, the DNA-binding domain (located between amino acids 96 and 286), is responsible for sequence-specific DNA binding [4]. Recently, an additional functional domain was identified on p53 between the transactivation and the sequence-specific DNA binding domains. Walker and Levine [5] localized a region between amino acids 61 and 94 that is necessary for efficient growth suppression. The tetramerization domain (amino acids 319-360) of p53 interacts with other p53 protein chains to form the biologically active tetramer, as was shown by peptide mapping [6]. In reality this is not a true tetramer, but a dimer of dimers in which each chain contributes with a β-pleated sheet and an α-helix [7-9]. The fifth domain (basic domain) carries a highly positive charge and interacts non-sequence specifically with DNA. This basic region is located at the C-terminus between amino acids 363 and 386 [10,11]. p53 lacking the last 30 amino acids at the C-terminal end exhibits higher double stranded DNA-affinity, suggesting a negative regulation of specific DNA binding by the basic region [12,13], but this same truncation activates binding to damaged DNA structures [14,15]. Oligomerization is thought to be influenced by the C-terminal region including the basic domain [16]. The flexible regions between the domains are involved in conformational changes necessary for the molecule's regulatory effects. Changes in the primary or secondary structure of p53 results in protein variants with altered biological characteristics.

As single point mutations modify p53's structure and function, post-translational modifications are expected to interfere with transcriptional control as strongly. The most prominent of these post-translational modifications is the phosphorylation of p53. Phosphorylation sites span the entire protein and distinct phosphorylation sites are found in at least three domains and in the flexible segments between them. The importance of phosphorylation of p53 in determining the biological role of the protein is being increasingly appreciated [17,18]. It seems that the two flexible domains, the N-terminal transactivation domain and the C-terminal basic domain, are the major sites of protein kinase activity. Incidentally, these are the regions of p53 that induce most of the anti-p53 autoantibodies in cancer patients [19].

The most useful reagents for the detection and quantification of proteins secreted by cells or present in tissues are monoclonal antibodies (mAbs). Indeed, mAbs and polyclonal antibodies are the reagents of choice to characterize the active or inactive status of tumor suppressor p53 [20-23]. Specific DNA binding of p53 was greatly increased after saturation with an antibody termed 421. This antibody blocked the C-terminal non-specific DNA-binding sites, favoring the specific DNA-binding of the middle region of the protein [13]. Native p53 (wild-type or mutant) in cell lines and tissues is a weak immunogen, probably because of the low level of protein expression. Although a number of anti-p53 antibodies are available commercially, their applicability is often limited. Lane and coworkers [24] conducted a thorough analysis of the epitope distribution of p53, and concluded that in addition to the immunogenic N- and C-termini, very few new antigenic sites can be identified. Based on their recommendation, new approaches will have to be employed to identify novel immunological reagents to p53. According to these

authors, immunization with peptides may be the most productive route to overcome the host restraints and to isolate mAbs to hidden p53 epitopes.

Here we report the generation of a highly sensitive mAb to p53 by using a double phosphorylated peptide immunogen corresponding to amino acids 371-393 of the C-terminal basic domain of p53. In conventional aqueous environments, mAb p53-18 was highly specific for phosphorylated Ser378 and Ser392 at both the protein and the corresponding peptide levels. When the peptide conformation was changed during the assay procedure to that of an α-helix, however, mAb p53-18 also detected the unphosphorylated p53 C-terminal fragment. The immunodominance of the phosphorylated p53 C-terminus is indicated by the fact that cancer patients' sera preferentially labeled the same sequence as mAb p53-18, i.e., the double phosphorylated peptide antigen.

Materials and Methods

Synthetic peptides

Peptides were synthesized on Milligen 9050 and Rainin PS3 automatic synthesizers using 9-fluorenylmethoxycarbonyl (Fmoc) amino acids according to standard procedures [25]. Phosphoserine residues were incorporated as Fmoc-Ser(PO_3HBzl)-OH [26], purchased from Novabiochem, Ltd. (San Diego, CA, USA). Peptides and phosphopeptides were detached from the solid support with trifluoroacetic acid (TFA) and they were purified by reversed-phase high performance liquid chromatography (RP-HPLC) using an aqueous acetonitrile gradient elution system containing 0.1% trifluoroacetic acid as an ion pairing reagent. The integrity of the peptides and phosphopeptides was verified by mass spectroscopy. Table I lists the sequences of the synthetic peptides and Table II demonstrates the methods used for their characterization. Two sets of peptides were made. One of them contained peptides of 23 amino acids long, and the second set contained peptides of 33 amino acids long (the peptides were extended to their N-termini). The peptides were either unphosphorylated, contained single phosphate groups on Ser378 or Ser392, or phosphates on both serines.

Immunization

The 23-mer double phosphorylated peptide was co-synthesized with a turn inducing spacer (Gly-Ala-Gly) and the immunodominant T-helper cell determinant 31D of the rabies virus nucleoprotein [27] in an orientation that placed the T-cell epitope to the N-terminus and the B-cell epitope to the C-terminus. Groups of six week-old female C3H/He mice were inoculated with 10 μg of the tandem peptide mixed with 50% complete Freund's adjuvant in each hind leg. Fourteen days later,

the mice received a booster immunization of 20 μg in 50% incomplete Freund's adjuvant. Ten days after the booster immunization, the mouse sera were screened and a third immunization was applied, followed by fusion of splenocytes with myeloma cells five days later using previously described methods [28]. Hybridomas were grown in selective medium, screened and subcloned according to standard procedures [29]. High titer of mAbs were produced as ascites upon injection of hybridoma cells into immunodeficient RAG-2 mice. The isotypes were determined using the Calbiochem (San Diego, CA, USA) isotyping kit according to the manufacturer's specifications.

Affinity purification of baculovirus expressed murine p53

Ascites of mAb p53-18 was precipitated using saturated ammonium sulfate and the pellet was dialyzed against phosphate-buffered saline (PBS) pH 7.6 containing 0.1 M NaCl. The mAb was purified by size-exclusion chromatography on a Sepharose S-300 column and fractions 11-17 (7 ml each) containing IgM were collected and analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fractions 11-14 containing IgM immunoglobulin were pooled. Ten mg of purified mAb p53-18 was coupled to 2 ml of CNBr-activated Sepharose 4B Fast Flow (Pharmacia, Uppsala, Sweden). Unreacted functional groups on the matrix were blocked by incubation with 0.1 M Tris-HCl buffer, pH 8.0. The column was washed several times alternating with Tris-HCl buffer, pH 8.0, and acetate buffer, pH 3.6. The source of murine p53 protein was a cellular extract of Sf9 insect cells infected with recombinant baculovirus expressing murine wild-type p53 generated by the Expression Vector Facility of the Wistar Institute. Sf9 cells were collected, lysed in PBS containing 0.1% NP-40 and centrifuged at 12,000 ×*g* for 15 min. The column was equilibrated with PBS, pH 7.6, containing 0.1 M NaCl. The supernatant containing soluble p53 was loaded onto the p53-18 affinity column followed by

washing with phosphate buffer and PBS to elute non-specifically bound proteins. Murine p53 protein was recovered from the affinity column by elution with 0.1 M glycine, pH 2.9, into tubes containing 1/10 fraction volume of 1 M Tris-HCl, pH 8.0. The protein composition of each fraction was evaluated using 12% SDS-PAGE in the presence of protein molecular mass markers and visualized with Coomassie Blue staining.

Western blot

Fractions from the immunoaffinity purification of murine p53 as well as p53 variants expressed in various cells (see results and legend to Fig. 3) were also probed with mAb p53-18 by using conventional Western blot techniques [29]. Briefly, proteins and protein fractions were applied to a 12% SDS-PAGE gel and after electrophoresis were transferred to nitrocellulose membranes. The membrane was blocked with 5% fat-free milk dissolved in PBS containing 0.01% Tween 20. After washings, the membranes were incubated with various dilutions of the antibody. ^{125}I-labeled (400,000 cpm/ml) goat anti-mouse IgG was used as a secondary antibody in PBS containing 0.01% Tween 20. Membranes were washed extensively and autoradiography was performed with X-Omat X-ray films (Kodak, Rochester, NY, USA).

Recombinant p53 protein

Recombinant p53 cDNAs were cloned into the bacterial expression vector pT5T encoding an additional N-terminal 6-histidine epitope tag. Recombinant proteins were expressed in *E. coli* BL21. Bacteria were lysed in an extraction buffer and bacterial DNA was degraded by addition of 50 µg/mL of DNase I. The suspension was cleared by centrifugation at 120,000 × g at 4°C, and p53 protein in the

supernatant was purified over a metal-chelating column (Pharmacia, Piscataway, NJ, USA) according to the instructions of the manufacturer.

Enzyme-linked immunosorbent assay (ELISA)

Direct ELISA was performed as described previously [30]. First the active dilution range of antibody preparation was determined, and then the selectivity of a given antibody dilution toward various peptide antigens was assessed. Generally 40 ng – 2.5 μg peptide antigens and 0.5 ng – 1.2 μg protein samples were loaded in each well. Actual peptide concentrations were determined by RP-HPLC [31]. Because mAb p53-18 is an IgM subtype, the ELISA conditions were modified as follows: The peptides were applied to the plate and allowed to dry overnight. The plates were washed with a PBS solution, pH 6.8, containing 0.04% Triton X100. This solvent was used in all consecutive steps. To look for conformational effects, the assay was repeated identically, except the peptides were dissolved in trifluoroethanol (TFE) instead of water and dried to the ELISA plate overnight [32]. Sera of cancer patients and healthy controls were screened for circulating anti-p53 autoantibodies using the same protocol. This time the washing buffer contained 1 mg/ml of bovine serum albumin and the secondary antibody was a sheep anti-human IgG. The rest of the ELISA protocol remained unchanged.

Liquid chromatography-coupled mass spectroscopy (LC-MS)

The affinity purified mouse p53 protein was digested with trypsin at 37°C for 6 h in 50 mM ammonium bicarbonate, pH 8.5. The reaction was halted by

lyophilization of the sample. The tryptic digest was submitted to LC-MS as follows: RP-HPLC was performed by using a Hewlett-Packard model 1100 binary system and a Vydac C18 narrow bore column with 0.1% aqueous trifluoroacetic acid and 0.1% trifluoroacetic acid in 90% aqueous acetonitrile as solvents at 35°C. A 1.25 %/min gradient was applied with the system running at a 0.3 mL/min flow rate. Peaks were recorded at 214 nm. MS detection was achieved using a VG Biotech BIO-Q triple quadrupole mass spectrometer operating in the positive ion electrospray ionization mode in the 250 – 950 m/z scan range, 46 V cone voltage at 80°C. The instrument was calibrated with CsI prior to the LC-MS.

Results

The double phosphorylated 23-mer peptide was co-synthesized with a sequence containing an immunodominant T-helper cell epitope delineated from the rabies virus nucleoprotein. This chimeric peptide construct was injected into mice to generate monoclonal antibodies. An earlier application of this strategy using a B-cell epitope of rabies virus has resulted in the production of antibodies capable of providing protective immunity against a lethal rabies virus challenge infection [28]. After fusion of the spleen cells of mice immunized with the p53-phosphopeptide construct, eight hybridomas were selected. All of these clones recognized the immunizing double phosphorylated peptide antigen strongly. The most promising mAb, p53-18 showed higher binding than antibodies from the other hybridomas. Antibody isotyping revealed that mAb p53-18 was an IgM. IgMs are known to bind to a number of unrelated proteins on ELISA. Therefore, in all consecutive assays we used thorough washing of the plates with 0.04% Triton X100 in PBS solution instead of blocking the plates with horse serum or other proteins or mixtures of proteins after application of the antigens.

Hybridoma cells secreting antibody p53-18 were subcloned and the mAb was produced as ascites fluid in RAG-2 mice. First, the antigen specificity of mAb p53-18 was characterized by binding to the 23-mer peptides. One μg each of the four peptides, unphosphorylated, phosphorylated on Ser378, phosphorylated on Ser392 or phosphorylated on both Ser378 and Ser392 were tested against mAb p53-18 using serial dilutions of the ascites fluid ranging from 1:100 to 1:10000 (Fig. 1). The double phosphorylated peptide was recognized at an ascites dilution as high as 1:2000. The antibody binding constantly increased with decreasing dilution. None of the other three peptides were recognized below a 1:500 antibody dilution, and the recognition

of the double phosphorylated peptide was considerably stronger than any of the other three peptides in the entire antibody dilution range. If any distinction among the unphosphorylated and the two monophosphorylated peptides is to be made, the Ser392 phosphorylated peptide appeared to be slightly better recognized than the other two variants. This experiment was repeated with the four 33-mer peptides, and with a constant dilution of the ascites fluid (1:200) with the antigen load varying between 0.004 and 5 μg. Again, only the double phosphorylated peptide bound appreciatively to mAb p53-18 (data not shown).

After determining the peptide specificity of mAb p53-18, we investigated whether binding of mAb p53-18 was similarly dependent upon the presence of phosphate groups at the p53 protein level. Two p53 protein preparations were tested by direct ELISA using a 1:200 dilution of the ascites fluid. One of the p53 proteins (corresponding to the human sequence) was expressed in *E. coli*, and is likely to be unphosphorylated, or phosphorylated to a very low degree. The other protein variant was expressed in insect cells infected with mouse p53 baculovirus recombinant, and was expected to be highly phosphorylated. As a negative control, protein E7 of human papilloma virus (HPV)-16 was expressed by the baculovirus system using identical conditions. From the two p53 variants, the mAb recognized only the phosphorylated protein (derived from insect cells), and failed to bind to the unphosphorylated variant (derived from *E. coli*) (Fig. 2). Actually, the mAb binding to the phosphorylated protein exhibited some "pro-zone" binding behavior, indicating optimal antigen-antibody interactions during these experimental conditions. Positive binding was detected with as little as 0.08 μg antigen. It needs to be mentioned that some unspecific binding to the E7 protein was also observed, but this was well below the binding to p53 and without the "pro-zone" binding characteristics. Antibody p53-18 did not cross-react with the unphosphorylated

protein variant even when as much as 0.3 μg protein was loaded. For a positive control, we used the commercially available antibody 421. It is considered that antibody 421 is a weak reagent in ELISA. Nevertheless, in our hands using identical assay conditions, the same *E. coli* expressed p53 protein preparation (0.25 μg and higher amounts) was recognized by a 1:50 dilution of polyclonal antibody (pAb) 421 (Fig. 2). Antibody 421 binds to an unmodified (unphosphorylated and unglycosylated) epitope around Ser378 [33]. Our peptide ELISA results supported this finding, as we detected low levels of 421 binding (at antibody dilution of 1:20) to 1 μg of the unphosphorylated 23-mer peptide (O.D. = 0.25) and complete lack of antibody 421 binding to the Ser378 phosphorylated or double phosphorylated peptides (data not shown). Nevertheless, because of the low degree of 421 binding to the peptides, these results need to be treated with caution. In summary, the combined peptide and protein ELISA data clearly show that mAb p53-18 recognized p53 protein and its fragments regardless of the species of origin (*i.e.*, human or mouse), but only if the C-terminus of the protein was phosphorylated.

In the next step, we characterized the ability of mAb p53-18 to bind p53 on Western blot (Fig. 3A). The antibody bound to mouse p53 expressed in insect cells infected with baculovirus, in a pattern identical to that obtained by using another commercially available mAb, antibody 240 (Fig. 3B). Antibody 240 recognizes an amino acid stretch around residue 215, and labels p53 from many species, e.g. human, mouse, rat, etc. [34]. Antibody p53-18 also reacted with different forms of p53, including p53 MD (MI234, EG168) and p53 VD (AV135), mutants of mouse p53 with amino acid exchanges within the mutational hot spot domain (expressed by vaccinia virus in infected cells). In addition, the antibody cross-reacted with human wild-type p53 expressed by adenovirus in 293 cells, yet it failed to label the negative controls, such as insect cells infected with baculovirus alone, human

immunodeficiency virus gp160 expressed by vaccinia virus and rabies glycoprotein expressed by adenovirus in 293 cells. Again, the recognition pattern of mAb p53-18 was basically identical to that of mAb 240 to all positive and negative control protein variants (Fig. 3). When an extract of 4×10^5 293 cells was loaded p53 protein could not be detected with either the test or the control antibodies (lanes 8) although 293 cells may express endogenous p53. A possible explanation is that p53 is actively degraded in adenovirus infected cells through a mechanism which involves association with the large E1B protein [35].

MAb p53-18 was precipitated from the ascites with ammonium sulfate and was purified by size-exclusion chromatography (Fig. 4A). The purity of the antibody fractions were analyzed by SDS-PAGE under reducing conditions. Fig. 4B shows the SDS-PAGE separated fractions 12-14, containing mAb p53-18, visualized with Coomassie Blue staining. In these fractions, only two bands, one corresponding to the heavy chain, and one corresponding to the light chain could be detected on the gel demonstrating that mAb p53-18 was separated from other proteins present in the ascites fluid. The purified antibody was used to prepare an affinity column on which murine p53, expressed in Sf9 insect cells was isolated (Fig. 5). The fractions eluted with glycine buffer pH 2.9 were analyzed with SDS-PAGE. Staining of the gel with Coomassie Blue evidenced the presence of pure p53 protein in fractions 1-4. These indicate that mAb p53-18 (unlike some other anti-p53 antibodies) can be used to specifically detect p53 protein in Western blots, and immunoaffinity columns containing mAb p53-18 will be able to isolate C-terminally phosphorylated p53 originated from various sources.

After purification of mouse p53 from the recombinant baculovirus infected insect cells, we investigated whether or not the isolated protein was indeed phosphorylated on the PKC site (Ser375) and the CKII site (Ser389). To this end, the

immunoaffinity purified p53 protein was subjected to tryptic digestion and LC-MS. The chromatographic profile obtained following the tryptic digest showed a typical pattern of peaks eluting between 10 – 40 min after application of the gradient. Location of the anticipated phosphorylated peptides is illustrated in Figures 6 and 7. These figures show the ultraviolet absorbance profiles and the selective ion profile for m/z 669 corresponding to the singly charged form of the phosphorylated peptide fragment, Val385-Asp390 (Fig. 6) and the selective ion profile for m/z 358, corresponding to the doubly charged form of the phosphorylated peptide fragment, Gly371-Arg376 (Fig. 7). Figures 8 and 9 illustrate the electrospray ionization mass spectra recorded in the chromatographic regions highlighted by the selective ion profiles. Both spectra clearly identified the anticipated phosphopeptide signals. The spectra also contained other major ions which may be due to additional, closely eluting sample components. The mass difference between some of these ions suggests these may be derived from an ethoxylate polymer, a contaminant often observed after immunoaffinity purification of proteins.

Similar treatment of the data to try and locate the corresponding unphosphorylated peptides produced a selective ion chromatogram for the m/z 635 at 25.4 min (data not shown). As expected based on earlier phosphopeptide chromatographic studies [36], this peak eluted later than that of the appropriate phosphopeptide. The mass representing this HPLC peak was consistent with the singly charged form of the unphosphorylated peptide fragment Gly371-Arg376. Analysis of the data for the other potential unphosphorylated peptide fragment, Val385-Asp390, failed to reveal any significant component of this mass. All of these data indicated that the protein produced by the baculovirus system in Sf9 insect cells and purified by using the mAb p53-18 immunoaffinity column was indeed phosphorylated at both the PKC and the CKII sites. The sample submitted to the

mass spectroscopy contained some unphosphorylated version at the PKC site. This can be potentially due to a smaller amount of partially phosphorylated protein variant copurifying with the fully phosphorylated version (compare antibody specificity with Fig. 1). Another explanation for the appearance of this unphosphorylated peptide fragment is phosphate loss during the isolation, digestion and chromatographic processes. Nevertheless, the CKII site remained completely phosphorylated even after all of these tedious sample manipulations.

In a separate study, we examined the conformation and non-sequence specific DNA-binding ability of our p53 C-terminal peptides and phosphopeptides [37]. While the peptides are mostly unordered in water, the unmodified peptide assumes a relatively strong α-helix conformation in the structure-inducing solvent TFE. The basic domain is predominantly unordered in the p53 protein, but it is located proximal to the tetramerization domain (amino acids 319-360; [6]), which forms an anti-parallel four helical bundle, determined by multi-dimensional nuclear magnetic resonance studies [7,8]. It is conceivable that the conformational changes in the basic domain, due to amino acid mutations, will affect the efficiency of tetramer assembly and function of p53. In order to determine whether mAb p53-18 saw a conformation-dependent epitope, we repeated the ELISA of the unphosphorylated and the double phosphorylated 23-mer peptides by using a conformation-sensitive assay protocol. During these assay conditions, the peptides are applied to the ELISA plate from TFE, and the antigens are allowed to dry [31]. The peptides interact with the plastic surface by exposing new hydrophobic and hydrophilic areas, and their structure presented by the solvent remains conserved causing a remarkable variation of peptide recognition by anti-protein antibodies [31,38]. By using this ELISA protocol, the diphosphorylated C-terminal p53 peptide was recognized at high levels by mAb p53-18, regardless of the secondary structure of

the peptide antigen (Fig. 10). The "pro-zone" binding behavior indicated optimal antibody-antigen interactions in the entire 0.04 – 2.5 μg peptide load range studied (the dilution of the ascites fluid was 1:1000). In contrast, the unphosphorylated peptide, unrecognized by the antibody during regular ELISA conditions, bound to the antibody only when the peptide was plated from TFE (Fig. 10). Nevertheless, this induced antibody binding remained at a level one magnitude below that of the diphosphorylated analog, and did not show "pro-zone" binding behavior in the studied antigen load range. These findings indicate that while the main feature of mAb p53-18 binding to peptide antigens is the presence of phosphate groups on Ser378 and Ser392, the antibody is also sensitive to antigen conformation. Antibody 421 is believed to recognize a linear epitope, and indeed, its binding to the same unphosphorylated peptide was neither increased nor significantly decreased when the peptide was plated from TFE (O.D. = 0.18; data not shown).

Finally, we tested the binding of the synthetic peptides to 40 sera of both cancer patients and healthy controls. First the sera, in 1:100 and 1:500 dilutions, were screened against the unphosphorylated, the Ser378 phosphorylated and the diphosphorylated 23-mer peptides. When the positive sera were identified, the specificity of the p53 autoantibodies to the presence or absence of the C-terminal phosphate groups was studied by serial dilutions of the sera (from 1:50 to 1:1500) against peptide antigens displaying all four possible phosphate forms and p53 protein produced in the baculovirus system. After four repetitions five sera could be identified that clearly bound to at least one of the peptides. From the five positive sera, two were originated from breast cancer patients (#7 and #15), two from cervical cancer patients (#31 and #38), and one from a healthy control (#27). When tested for peptide specificity, all five sera bound to the double phosphorylated peptide stronger than to any other phosphate isoforms (Table III). The four sera

from cancer patients also recognized the full-sized p53 protein. The serum from the healthy control subject did not recognize the p53 protein, indicating that the cross-reactivity with the double phosphorylated peptide was due to unspecific binding, or the epitope for serum #27 was not present in the protein preparation studied.

Discussion

Here we characterized a highly sensitive, multiphosphorylation-specific mAb to the C-terminus of p53. Most recently a polyclonal antibody, specific to phosphorylated Ser392 became marketed by New England Biolabs (Beverly, MA, USA). According to the manufacturer, this antibody was obtained by inoculation of rabbits with a C-terminal phosphopeptide conjugated to a carrier protein. This pAb is claimed to recognize p53 only after phosphorylation with CKII at Ser392. We compared the phosphate specificity of this pAb with our mAb p53-18 against p53 expressed in *E. coli* and the protein expressed in Sf9 insect cells infected by baculovirus, as well as against the four 33-mer peptides with the different phosphate forms. In our hands, the polyclonal antibody did not recognize the phosphorylated protein below 1 μg protein load. Actually, more definite binding was observed against the *E. coli* expressed protein up to 0.1 μg protein load. As a positive control on the same plate, mAb p53-18 bound to the p53 variant expressed in the baculovirus system more than twice as strongly than it bound to p53 expressed in *E. coli*, similarly to the pattern depicted in Fig. 2. At a peptide level, the Ser392 phosphate specific polyclonal antibody recognized the Ser392 phosphorylated and diphosphorylated long peptides weakly at an antibody dilution of 1:400 (Fig. 11). While complete loss of binding was observed at higher antibody dilutions, at lower dilutions the phosphate specificity seemed to be lost, explaining the detected recognition of the *E. coli* expressed protein variant.

Phosphorylation-specific mAbs capable of identifying various phosphate forms of p53 would be especially attractive in light of a recent report. Whole-cell proteins from control and apoptotic H460a cells were separated by two-dimensional electrophoresis and were transferred to nitrocellulose [39]. As a reporter antibody,

mAb BP53.12 was used to stain the p53 variants. Four isoforms of p53 differing in the phosphorylation state, determined by the electrophoretic mobility differences of the protein variants, could be detected by mAb BP53.12 in the control cells. Three additional isoforms were observed to be expressed at significant levels only in apoptotic cells [39]. Antibody BP53.12 recognizes the N-terminal domain of p53, where a large number of potential phosphorylation sites are found. As the phosphate specificity of this antibody is unknown, it is likely that some important phosphate forms of the antigens, unrecognized by the antibody, remain undetected in the system. Application of our immunization strategy to the N-terminal regions of p53 is likely to result in phosphate-specific mAbs to this domain; experiments that are currently in progress in our laboratories.

To determine the phosphate specificity of mAb p53-18 at the protein level, we used p53 expressed in two systems. To increase the production of p53 compared to mammalian tissues and cell lines, p53 is generally made in bacterial [12], baculovirus [40] and vaccinia virus [41] expression systems. The protein produced in *E. coli* is likely to be unphosphorylated, or phosphorylated at a low level. Bacterial environments for phosphorylation usually lack eukaryotic kinases, and this frequently prevents proper phosphorylation from occurring [42]. Indeed, p53 expressed in *E. coli* only binds DNA properly after interaction with cellular proteins, including various kinases [12]. In contrast, it has been shown that human p53 expressed in baculovirus-infected Sf9 cells displays a two-dimensional electrophoretic mobility pattern (and consequently phosphorylation pattern) identical to wild-type p53 from human cells [43]. Insect cells appear to contain all protein kinases necessary for phosphorylation of a mammalian protein [44], with at least 9 potential p53 sites phosphorylated in this system [43]. Accordingly, our murine p53 originated from the Sf9 cells was expected to carry phosphate groups on

both Ser375 and Ser389. Nevertheless, it is assumed that phosphorylation of p53 in the baculovirus system is heterogeneous [44], thus reducing the level of recognition by mAb p53-18 and allowing binding of pAb 421 [43]. Our LC-MS of the tryptic digest of mouse p53 produced by the baculovirus system clearly showed the presence of phosphate groups on both the PKC and the CKII sites. The highly discriminative properties of our mAb p53-18 towards the phosphorylation status of p53 will make this reagent suitable not only to detect low levels of phosphorylated p53, but in combination with other currently available antibodies, to estimate the actual degree of phosphorylation in the cell lines and tissues studied.

A recent report voiced concerns about the quality of anti-p53 antibodies [45]. According to this paper, immunohistochemical staining of tissue sections show remarkable differences when using different batches of pAb 421. Moreover, antibody 1801 from one source recognizes proteins in cell lines differently than the same antibody does that is obtained from another manufacturer [45]. These findings underline the importance of the thorough characterization of the anti-p53 antibodies. Here we described the antigen specificity of our mAb p53-18 in details unsurpassed in the current literature. However, the protein variants themselves often exhibit considerable variability. In the current paper, we presented our findings by using recombinant p53 produced by *E. coli*, purified by a metal-chelating column. We claim that this protein variant is unphosphorylated, or phosphorylated to a very low degree, as are most proteins obtained from bacterial sources. MAb p53-18 did not recognize this protein when we followed the ELISA protocol suitable for antibodies of the IgM isotype, and this finding mirrored those by using the unphosphorylated synthetic peptide. However, the antibody cross-reacted with another *E. coli* produced p53 preparation, purified from bacterial lysates by glutathione agarose affinity chromatography (marketed by Santa Cruz

Biotechnology, Santa Cruz, CA, USA). Here we would like to draw the attention of the variability of the currently studied protein preparations, and appeal for the thorough characterization not only of the antibodies but also of the distributed antigens. In this regard, fully purified synthetic peptides can be considered as reagents of significant utility.

Autoantibodies to p53 in humans, mostly in cancer patients, preferentially recognized the double phosphorylated peptide. Considering the relatively large distance of 14 amino acid residues between the two phosphate groups, it is reasonable to suppose that those three-dimensional structure variants of the protein, and perhaps of the peptide, in which phosphorylated Ser378 and Ser392 are close to each other in space, preferentially populate the conformational equilibria. This logic bestows a conformational nature on the epitope recognized by the anti-p53 C-terminal antibodies. In support of this, when the unphosphorylated peptide was forced to assume a more ordered structure of an α-helix it became recognizable by mAb p53-18. A helical wheel projection of the short peptide places Ser378 and Ser392 very close to each other (Fig. 12). In this regard, TFE might mimic a structure of the unphosphorylated antigen closer to the native conformation of the diphosphorylated peptide. Nevertheless the two structures are clearly different as we demonstrated a helix-breaking effect of phosphorylation (by using the 33-mer p53 C-terminal peptide) by circular dichroism and nuclear magnetic resonance spectroscopy [37]. This structural difference and the requirement for the phosphate group on the antigen for the recognition by mAb p53-18 is evident from the markedly decreased antibody binding to the unphosphorylated peptide even when both antigens were plated from TFE. Moreover, the unphosphorylated protein was not recognized, indicating that the very C-terminus of p53 does not populate α-helices. This is in accordance with the general expectations of conformational

preferences (actually the lack thereof) of extreme termini of proteins and circular dichroism studies of a large C-terminal synthetic fragment of p53 [46].

MAb p53-18 recognized both human and mouse p53 proteins. The differences between the C-terminal 23-mer human and mouse sequences are as follows: S371T, L383T, F385V, T387K and E388V. Careful examination of the sequences reveals no mutation within a 3 amino acid-distance from either phosphorylation site (Ser378 or Ser392). The unchanged primary structure around the phosphorylation sites explains the cross-reactivity of the antibody.

MAb p53-18 was obtained after immunization with the diphosphorylated C-terminal p53 peptide. Our original research plan was to select antibodies specific for either phosphate forms. An exhaustive analysis of the generated antibodies, however, revealed the complete lack of antibodies specific for Ser378 or Ser392 only. It appears that only the multiphosphorylated form of the C-terminal p53 domain is immunogenic enough to produce highly sensitive antibodies. This is not particularly surprising if we look at the analogy with the human τ protein. Abnormally hyperphosphorylated variants of the low molecular weight microtubule-associated protein τ are the main proteinaceous constituents of the paired helical filaments (PHF) of Alzheimer's disease [47]. PHF-τ carrying phosphate groups on nearby serines and threonines are highly immunogenic structures inasmuch as many anti-PHF antibodies, and all truly specific for the disease-related forms of τ, recognize multiphosphorylated antigens both at the peptide and the protein levels [38]. When mice were immunized with a chimeric 31D-multiphosphorylated τ peptide complex, the obtained sera were highly specific for the diphosphorylated antigen [48]. This observation further supports the idea that it is the extended presence of the protease-resistant multiphosphorylated

immunogens that can turn otherwise poorly immunogenic structures to highly immunogenic ones.

Phosphorylation of p53 at Ser392 appears necessary for tumor suppressor function since substitution with alanine abolishes the ability of p53 to suppress cell proliferation, whereas substitution with aspartic acid, which mimics phosphoserine, has only a partial effect on p53-mediated growth inhibition [49]. The suppressor function is a highly controversial issue. A recent paper claims that individual mutations of the N-terminal serines, as well as Ser315 and Ser392 to alanine or glutamic acid result in equivalent levels of transcriptional activation in standard transient transfection experiments [50]. However, when p53's transcriptional activation is measured in cells that attain G1 arrest upon contact inhibition, wild-type p53 is inactive, and only altering Ser392 to glutamic acid results in a functional protein. This Ser-Glu mutant also has an increased ability to bind DNA [50]. On the other hand, Fiscella and coworkers [51] reported that elimination of the Ser392 phosphorylation site has no discernible effect on p53 function, and phosphorylation is also reported not to affect the tetramerization of p53 [52]. Again, in contrast, most recent equilibrium ultracentrifugal analyses showed that phosphorylation of Ser392 increases the association constant for reversible tetramer formation nearly 10-fold [53]. The actual level of phosphorylation of the C-terminal sites are questionable because both PKC [54,55] and CKII [56,57] have to be translocated from the cytosol to the nucleus to actively phosphorylate p53. This gives rise to a possible difference between the status of phosphorylation in healthy or cancerous cells. Actually, murine equivalents of Ser371, Ser376, Thr377, and Ser378 of human p53 all can be phosphorylated by PKC in vitro, but loss of the pAb 421 epitope could not be detected in the cells suggesting that p53 and PKC may not interact at all in vivo [58]. Many of

these controversies can be solved by measuring the binding of mAb p53-18 to p53 variants in different cell compartments, cell types, and disease states.

In summary, here we described a novel phosphate-specific mAb to p53. This antibody bears the promise to be a highly useful biochemical marker to detect low levels of p53 protein in different tissues, and to be a key tool to characterize the status of phosphorylation of the C-terminus of p53 protein in various cell types, solution environments and stages of tumor progression.

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(Figure legends)

Fig. 1. Binding of mAb p53-18 to synthetic peptides corresponding to amino acids 371-393 of p53 protein. For these experiments, 1 μg amounts of the synthetic peptides were diluted in water and were applied to ELISA plates. Plates were blocked with Triton X100 dissolved at a concentration of 0.04% in PBS buffer, and the ascites fluid containing the antibody was added in a series of dilutions ranging from 1:100 to 1:10000. The open triangles and dotted line represent the unphosphorylated peptide; the open circles and dashes represent the peptide phosphorylated on Ser378; and the open squares and dots and dashes represent the peptide phosphorylated on Ser392. The closed symbols represent the double phosphorylated peptide; closed triangles and solid line correspond to the 378P, 392P peptide against mAb p53-18, and closed circles and randomly staggered line correspond to the same peptide tested for binding to an unrelated IgM control mAb.

Fig. 2. ELISA of phosphorylated and unphosphorylated p53 protein variants. Closed triangles and solid line represent p53 expressed by a recombinant baculovirus in insect cells against mAb p53-18. Crosses and dotted lines represent the negative control in which the unrelated E7 of HPV-16 protein was expressed by baculovirus and reacted against mAb p53-18. p53 expressed in *E. coli* is symbolized by circles. Closed circles and dots and dashes correspond to *E. coli* expressed p53 against mAb p53-18; open circles and dashes correspond to the same protein preparation against antibody 421. For the experiments with mAb p53-18 (IgM), the plates were blocked with Triton X100 after application of the antigens. Because pAb 421 is an IgG isotype, the plate was blocked with horse serum according to conventional ELISA protocols. The dilution of the ascites fluid containing mAb p53-18 was 1:200, and the supernatant of antibody 421 secreting cells was diluted to 1:50.

Fig. 3. Binding of mAbs to different p53 protein preparations on Western blot. Panel A corresponds to the blot developed with mAb p53-18; panel B corresponds to an identical blot developed with mAb 240. The antigens are as follows: lane 1: mouse p53 expressed by baculovirus in insect cells; lane 2: insect cell control infected with baculovirus alone; lane 3: p53 expressed by vaccinia virus in HeLa cells; lanes 4 and 5: mutated p53 variants: MD (MI234, EG168) and VD (AV135) expressed by vaccinia virus; lane 6: human immunodeficiency virus gp160 expressed by vaccinia virus; lane 7: human p53 expressed by adenovirus in 293 cells; lane 8: rabies glycoprotein expressed by adenovirus in 293 cells. The molecular mass markers are at the far right column.

Fig. 4. Size-exclusion chromatographic purification of mAb p53-18. The ammonium sulfate-precipitated antibody was applied to a Sepharose S-300 column in PBS, pH 7.6, containing 0.1 M NaCl. Panel A shows the elution profile. Protein peaks were identified by an ultraviolet detector operating at 280 nm. Panel B: The eluted fractions were analyzed by SDS-PAGE under reducing conditions, and proteins were visualized with Coomassie Blue staining. A set of BioRad prestained molecular mass markers is found at the far right column.

Fig. 5. Purification of murine p53 protein expressed by Sf9 insect cells infected with recombinant baculovirus using mAb p53-18 affinity column. The fractions from the column were analyzed by SDS-PAGE. The far left lane shows the position of the molecular mass markers. Lanes 1-8 are the fractions eluted with the glycine buffer, pH 2.9.

Fig. 6. Ultraviolet absorbance profile (A) and selective ion profile (B) representing the high performance liquid chromatogram of the tryptic digest of mouse p53

protein immunopurified with the p53-18 antibody column. The peak at 23.4 min corresponds to the peptide fragment with a m/z value of 669.

Fig. 7. Ultraviolet absorbance profile (A) and selective ion profile (B) representing the high performance liquid chromatogram of the tryptic digest of mouse p53 protein immunopurified with the p53-18 antibody column. The peak at 20.9 min corresponds to the peptide fragment with a m/z value of 358.

Fig. 8. Electrospray ionization mass spectrum recorded in the chromatographic region highlighted in Figure 6. The peak at m/z 669 corresponds to the singly charged form of the C-terminal phosphorylated peptide fragment Val385-Asp390. Chromatographic and spectroscopic conditions are found in Materials and Methods.

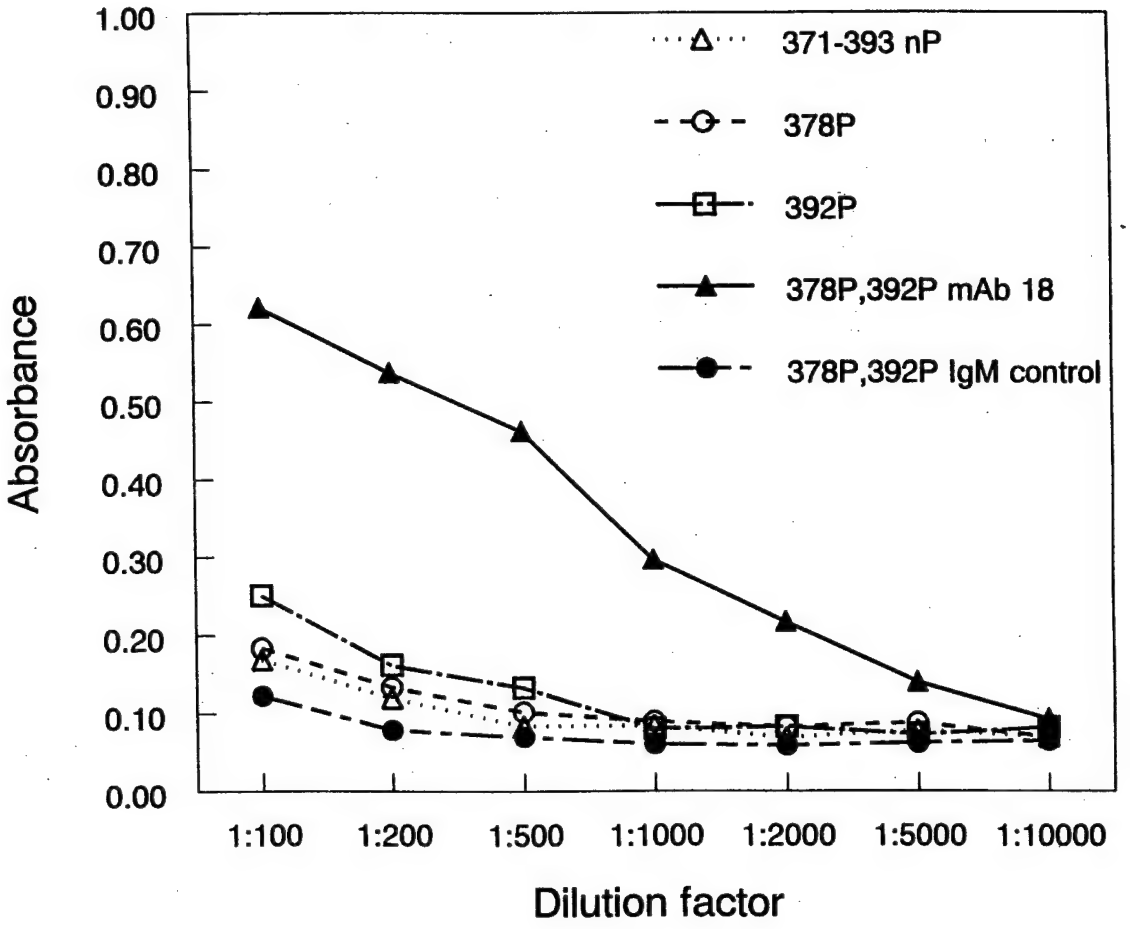
Fig. 9. Electrospray ionization mass spectrum recorded in the chromatographic region highlighted in Figure 7. The peak at m/z 358 corresponds to the doubly charged form of the phosphorylated peptide fragment Gly371-Arg376. Chromatographic and spectroscopic conditions found in Materials and Methods.

Fig. 10. Conformation-sensitive ELISA of the unphosphorylated and diphosphorylated p53 C-terminal peptides. In these experiments, 40 ng – 2.5 μg amounts of the peptides, dissolved in either water or TFE, were applied to the plate and dried overnight. The following day, the plates were blocked with Triton X100 and the ELISA was continued by using mAb p53-18 as the primary antibody. The unphosphorylated peptide is represented by triangles, and the diphosphorylated peptide is represented by circles. Open symbols correspond to peptides plated from water, and closed symbols correspond to peptides plated from TFE. The dilution of the ascites fluid containing the antibody was 1:1000.

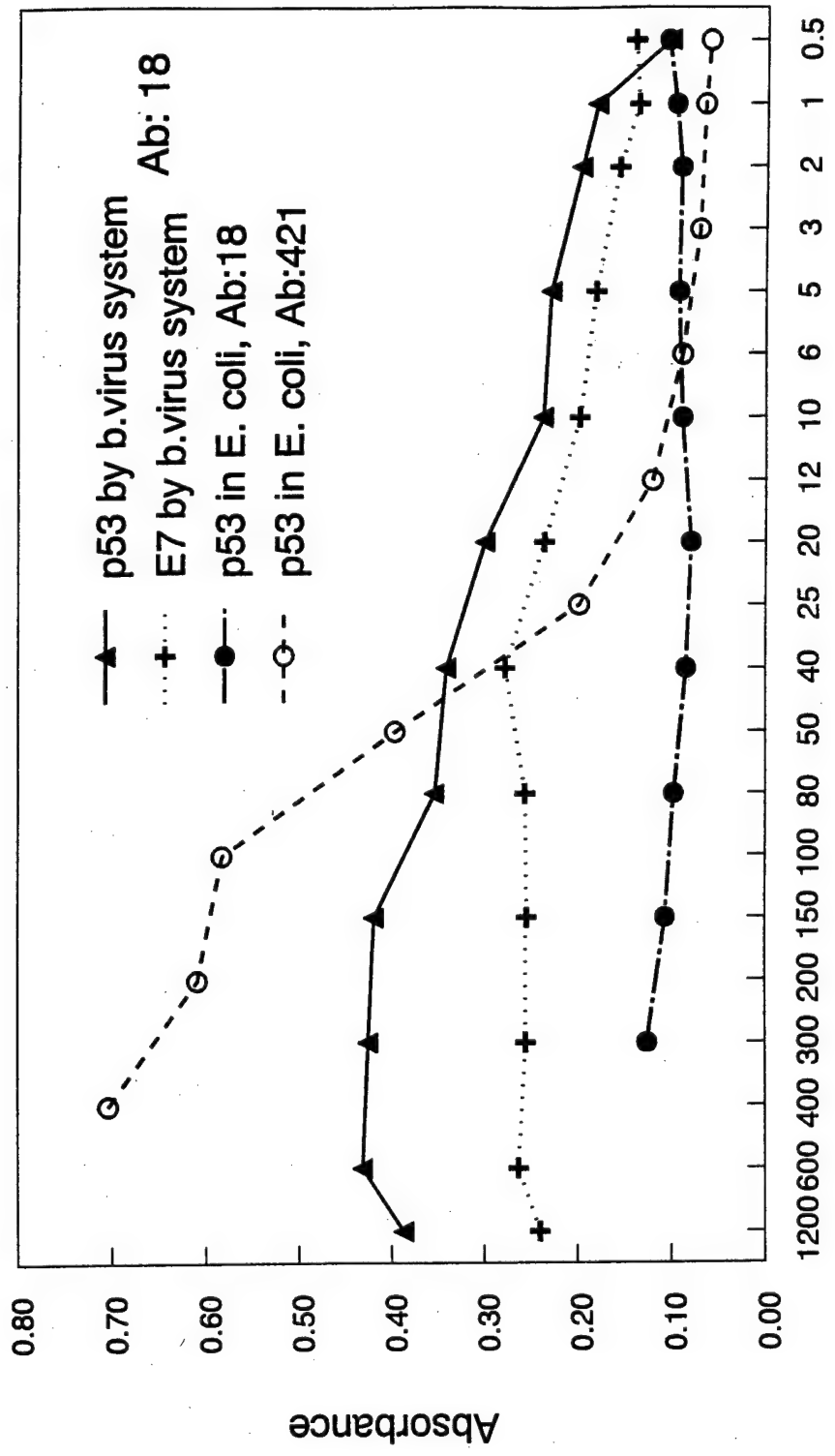
Fig. 11. Phosphate specificity of a pAb marketed by New England Biolabs, Inc. (Beverly, MA, USA). The pAb in various dilutions was probed against the four 33-mer peptides: open triangles and dots, unphosphorylated peptide; open circles and dashes, peptide phosphorylated on Ser378; open squares and dots and dashes, peptide phosphorylated on Ser392; full triangles and solid line, peptide phosphorylated on both Ser378 and Ser392. Conventional ELISA conditions were used.

Fig. 12. Helical wheel representation of the structure of p53 peptide 371-393. The inner circle corresponds to the first 18 amino acids, and the outer circle corresponds to amino acids 19-23, overlapping amino acids 1-5. Ser378 (in position 8) and Ser392 (at the outer circle in position 4) are marked with asterisks.

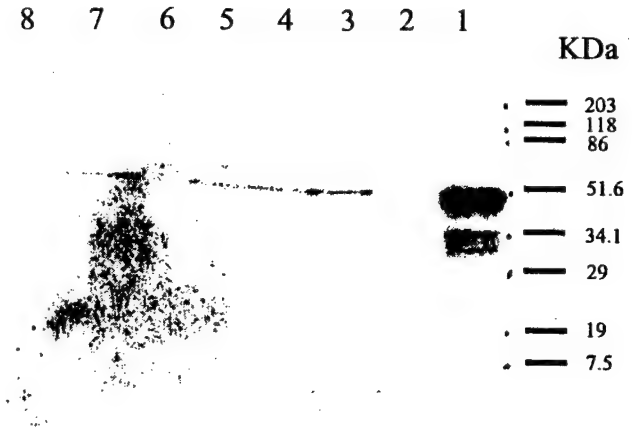
Otvos et al., Figure 1



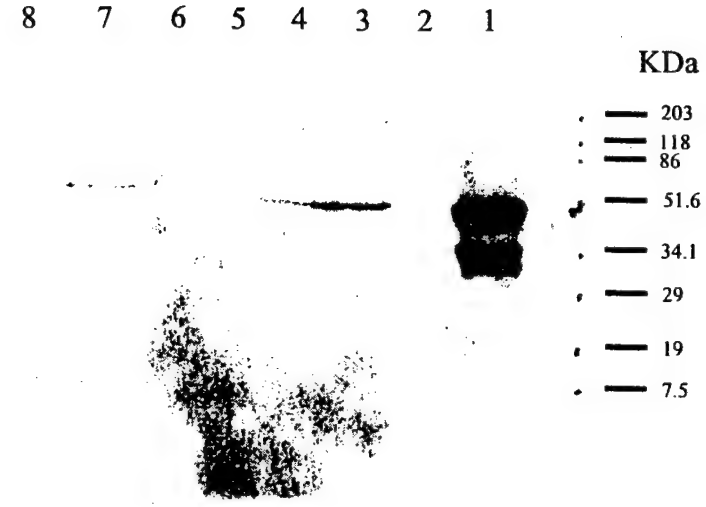
Otvos et al., Figure 2



Otvos et al.,
Figure 3

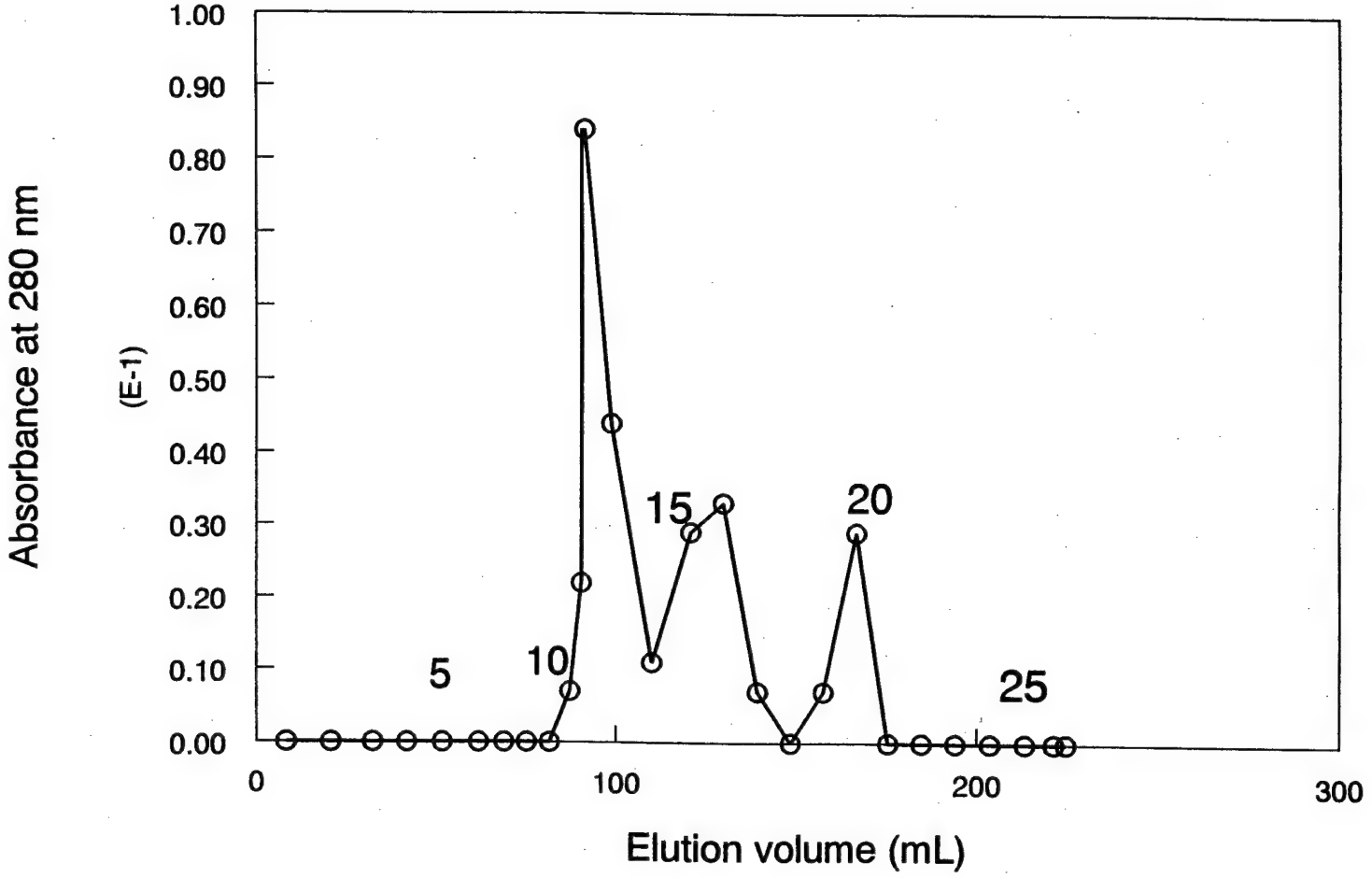


A: mAb p53-18

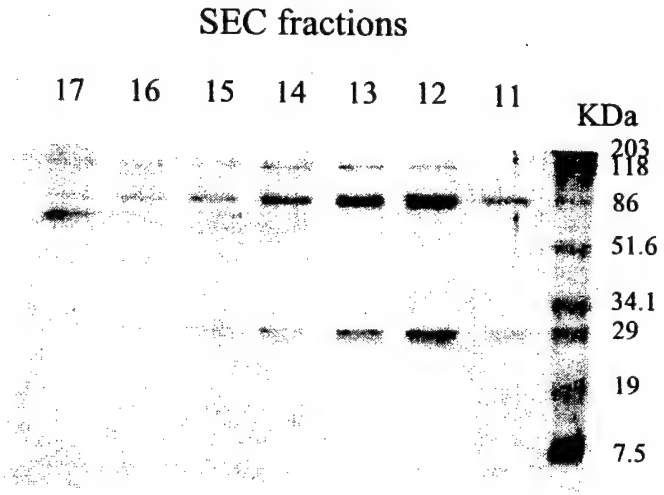


B: mAb 240

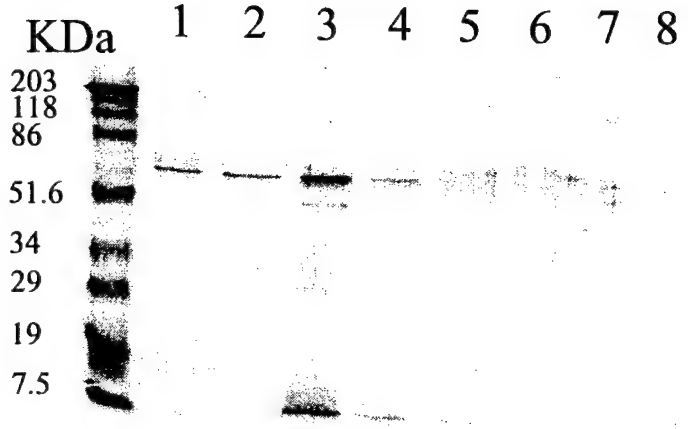
Otvos et al., Figure 4, panel A



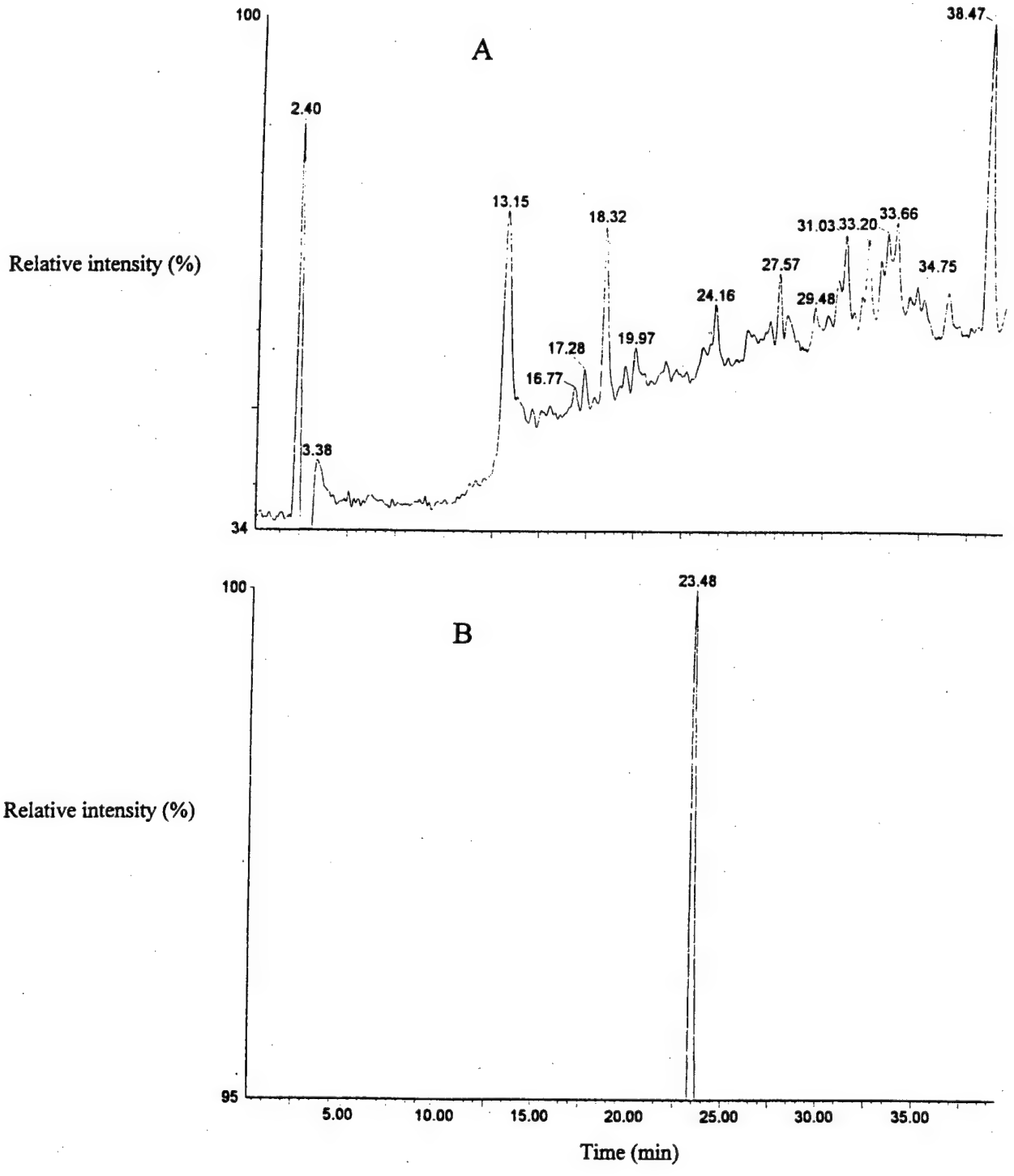
Otvos et al.,
Figure 4, panel B



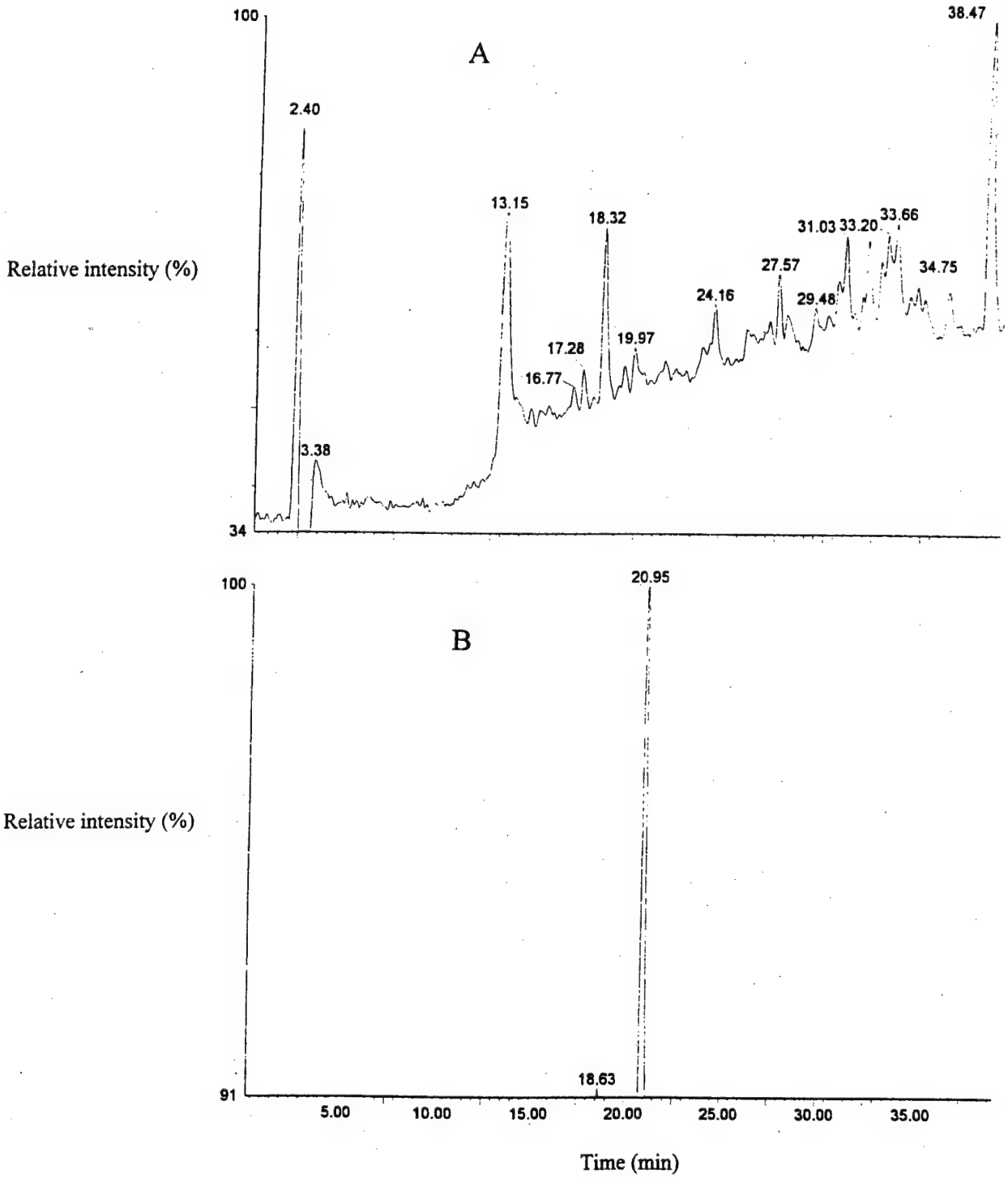
Otvos et al.,
Figure 5



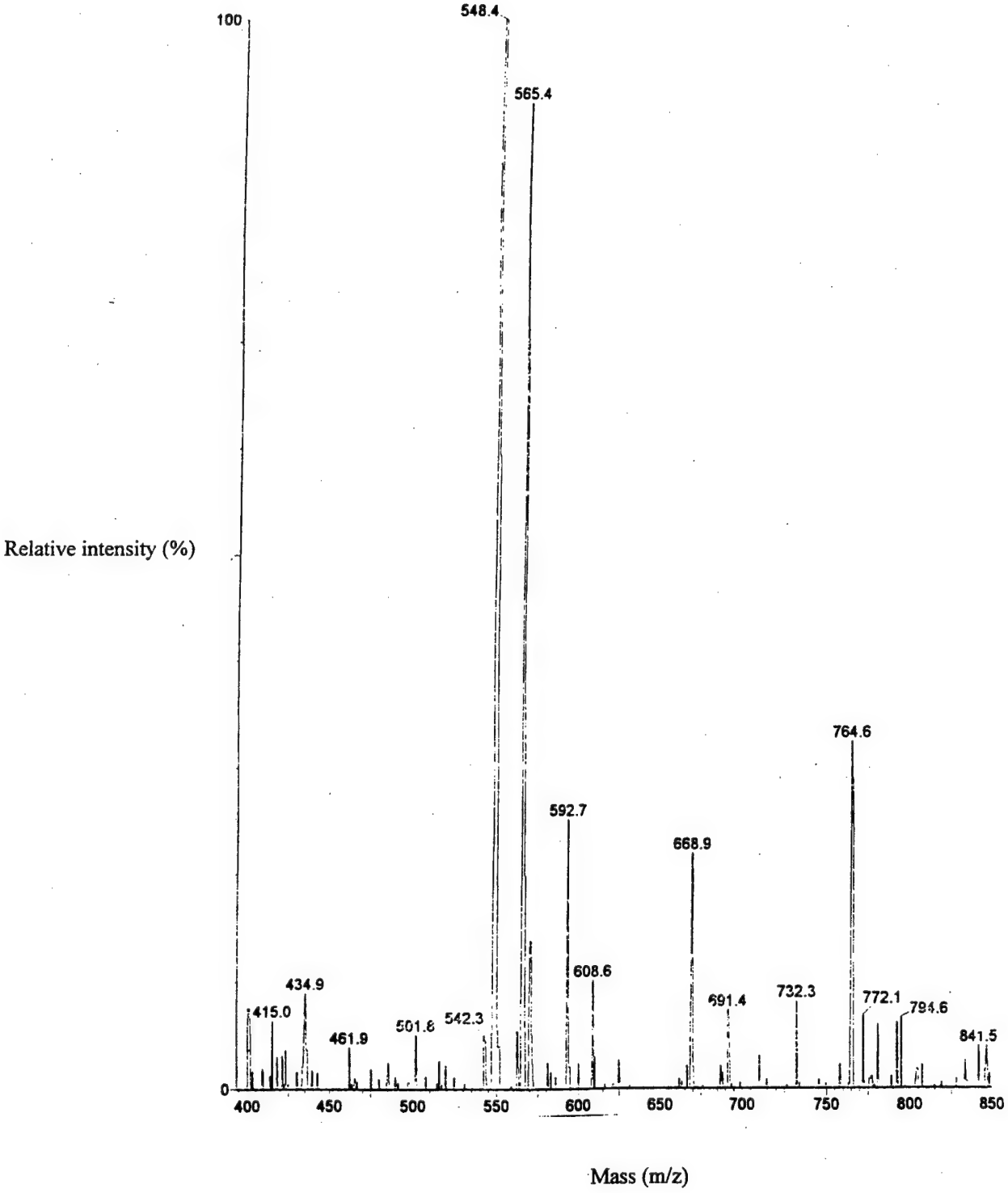
Otvos et al., Figure 6



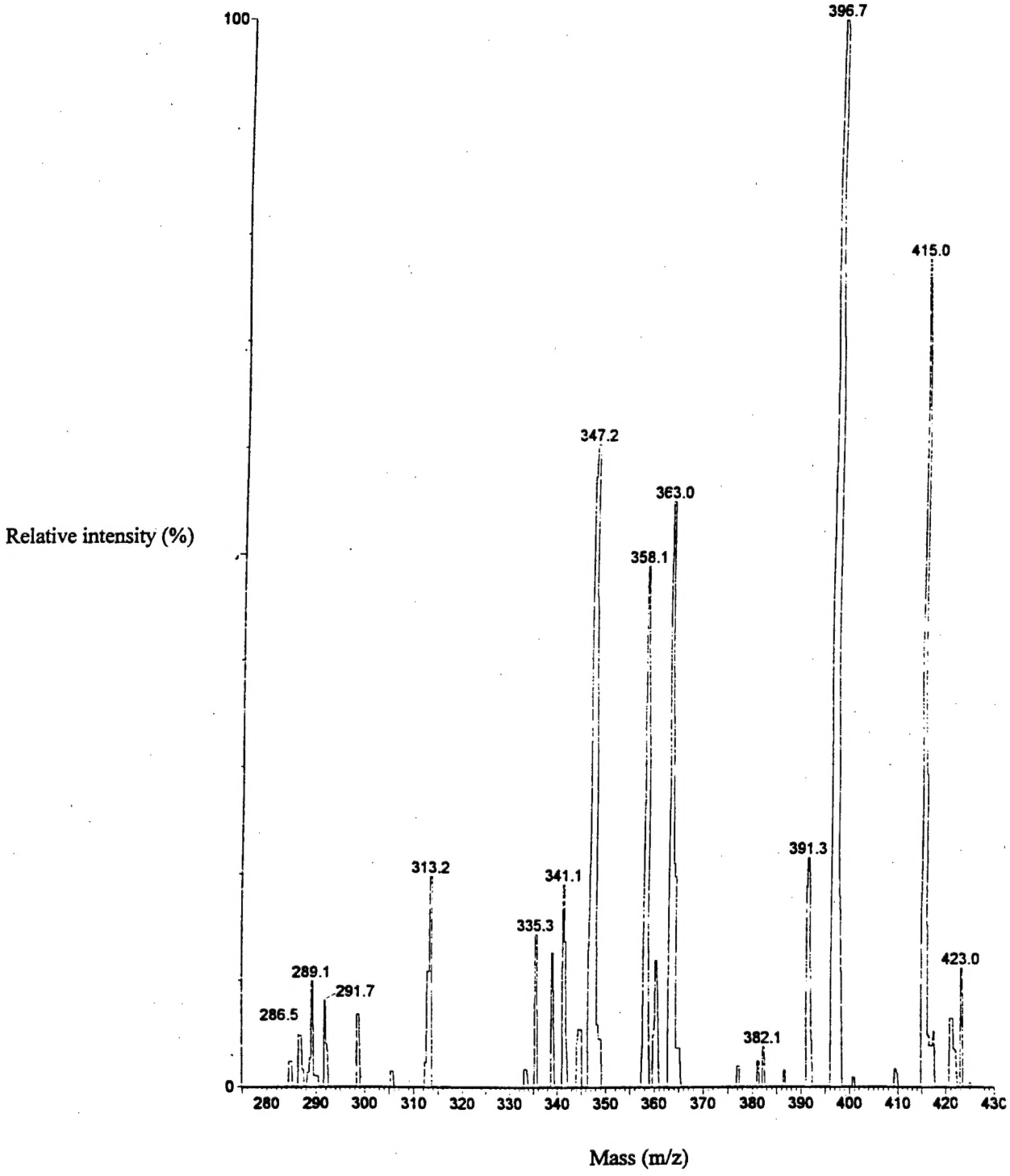
Otvos et al., Figure 7



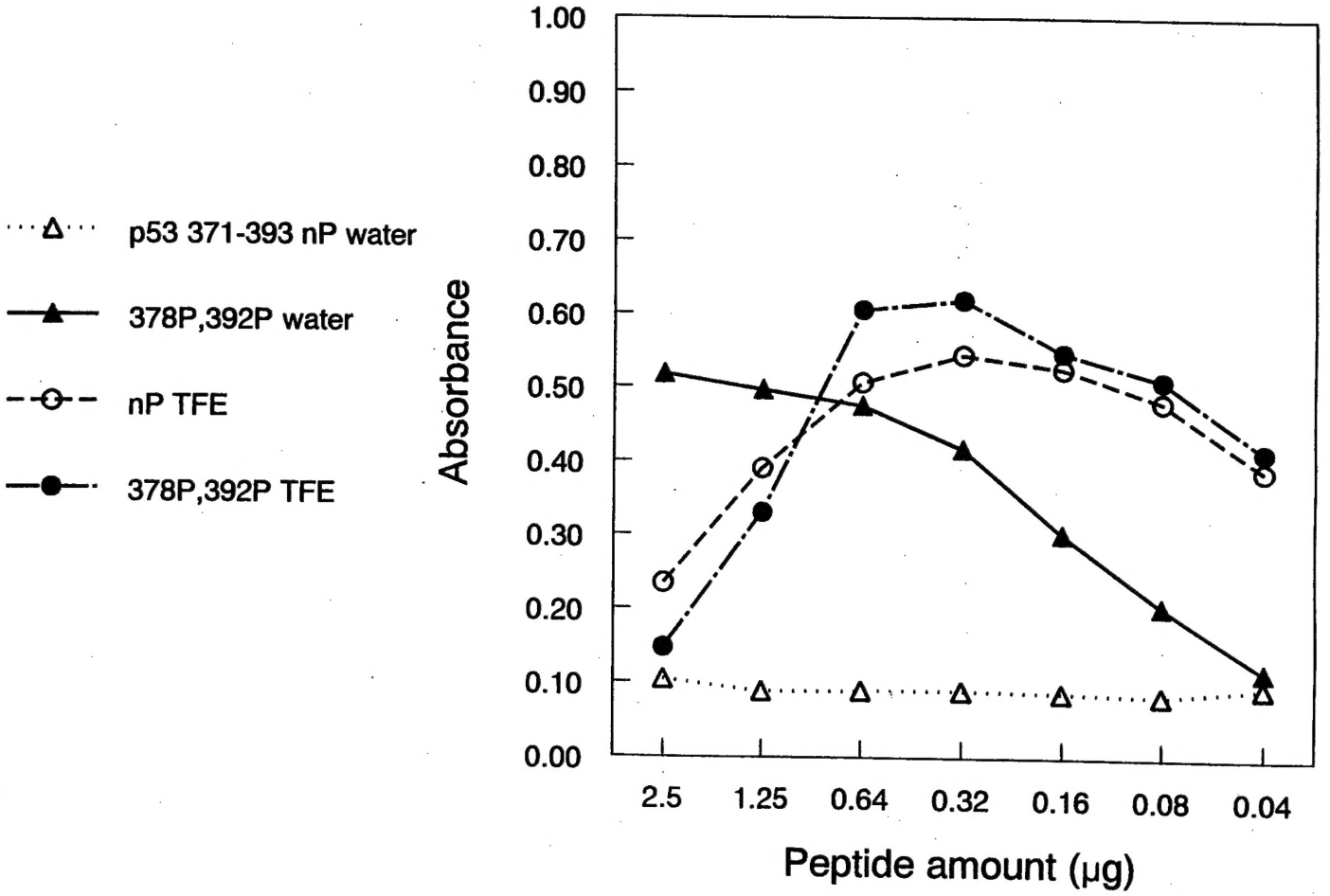
Otvos et al., Figure 8



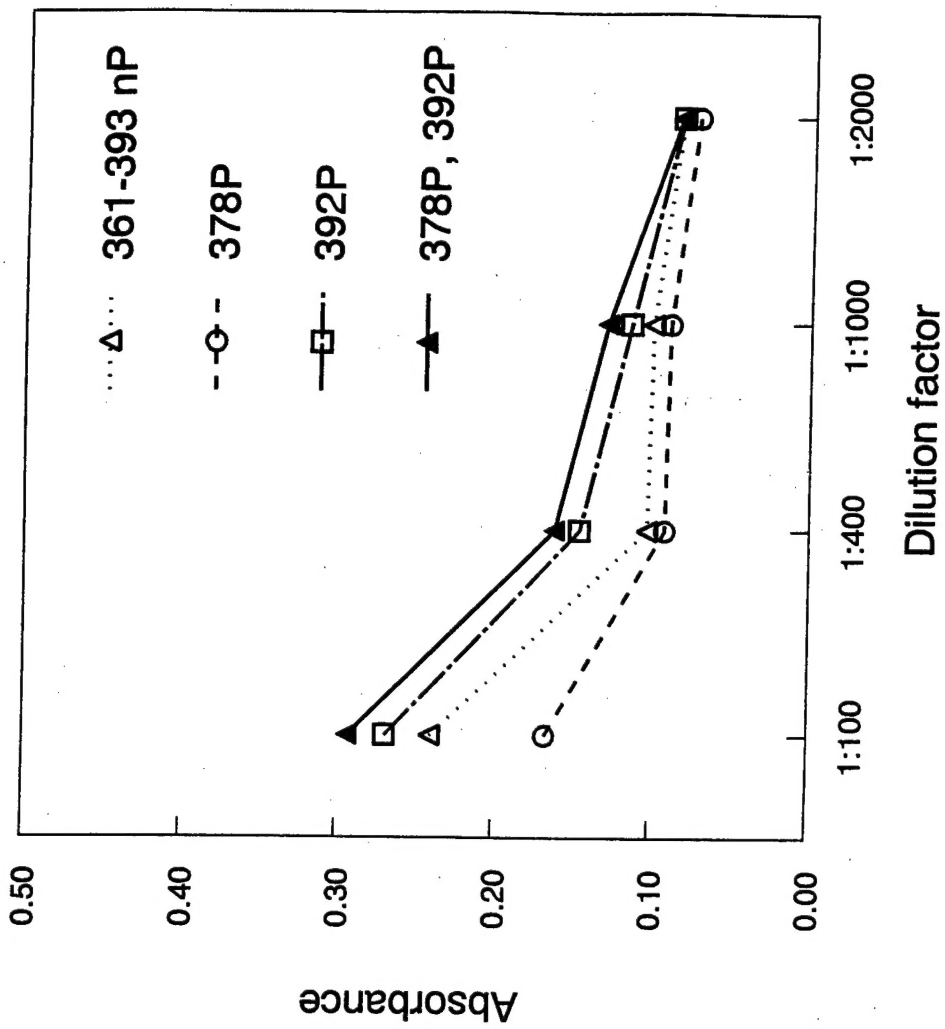
Otvos et al., Figure 9



Otvos et al., Figure 10



Otvos et al., Figure 11



Otvos et al.
Figure 12